AN ABSTRACT OF THE THESIS OF

Kelley Henderson for the degree of Master of Science in Chemical Engineering presented on December 3, 1991.

Title: Oxygen Mass Transfer and Shear Sensitivity Studies during Cultivation of *Nicotiana tabacum* var. Wisconsin 38 in a Stirred-tank Bioreactor.

Abstract approved *Redacted for Privacy*

Dr. Gregory Hoffer

Suspension cultures of *Nicotiana tabacum* var. Wisconsin 38 were cultivated in both shake flasks and a 2 L stirred-tank bioreactor. Growth rate, oxygen mass transfer and shear sensitivity of *N. tabacum* cultivation in the bioreactor was studied. Mass transfer studies were carried out by using a direct dynamic method for *in situ* estimates of both the volumetric respiration rate (Q_o) and the mass transfer coefficient (k_La).

Batch cultures grown in the bioreactor using a paddle blade impeller at 27 °C, 150 rpm, and 0.4 vvm did not grow as well as those cultivated in the shake flasks: maximum dry cell density in bioreactor was 4.12 g/L versus 11.51 g/L for shake flask. The growth curve representing cultivation in the bioreactor deviated from the shake flask following the onset of the exponential growth phase. Microscopic analysis of bioreactor culture samples revealed an increase in the number of cell fragments which was coupled to a decrease in the number of free

cells during the exponential growth phase. Other observable trends which occurred during the exponential growth phase were: a decrease in pH, an increase in dissolved oxygen, and a decrease in the respiration rate. These results indicated that *N. tabacum* var. Wisconsin 38 was sensitive to mechanical agitation during the exponential growth phase.

The dynamic model for *in situ* estimation of k_La was compared to the dissolved oxygen versus time data obtained from a pulse test on the aerating gas in the bioreactor. The model fit the data exceptionally, indicating that the estimate for k_La was adequate. Thus, this model is sufficient for estimating k_La in the culture as a function of process conditions for cultivation of plant cells in a stirred-tank bioreactor.

Oxygen Mass Transfer and Shear Sensitivity Studies during Cultivation of *Nicotiana tabacum* var. Wisconsin 38 in a Stirred-Tank Bioreactor

by

Kelley Henderson

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirement for the degree of

Master of Science

Completed December 3, 1991

Commencement June 1992

Approved:

Redacted for Privacy

Assistant Professor of Chemical Engineering in charge of major

Redacted for Privacy

Chemical Engineering Department Head

Redacted for Privacy

Dean of Graduate School

Date Thesis presented December 3, 1991

Typed by researcher Kelley Henderson

ACKNOWLEDGEMENTS

The author wishes to express her sincere appreciation to the following:

Dr. Gregory L. Rorrer for not only his help and encouragement through the course of this investigation, but also for his moral support through out the years as well.

Dr. Donald Armstrong for providing the initial callus cultures that were used in this investigation.

Vasja Marjanovic for donating his time and invaluable assistance in the lab.

Financial support during the time of research in the form of teaching assistantship and research assistantship was provided by the Department of Chemical Engineering, OSU Research Council, and NOAA.

NOMENCLATURE AND ABBREVIATIONS

A	=	Surface area available for oxygen mass transfer (m^2)
"A"	=	Indication of oxygen
C_A^*	=	Concentration of dissolved oxygen in equilibrium with the partial pressure of oxygen in the gas phase (mmole/L)
C_A	=	Dissolved oxygen concentration (mmole/L)
C_A^o	=	Initial dissolved oxygen concentration (mmole/L)
$C_{A,i}$	=	Measure dissolved oxygen concentration at time t_i (mmole/L)
$C_A(t_i)$	=	Predicted dissolved oxygen concentration at time t_i (mmole/L)
$C_{A,L}$	=	Bulk dissolved oxygen concentration (mmole/L)
D.O.	=	Dissolved oxygen
H	=	Henry's law constant (atm/mmole/L)
K_L	=	Overall liquid phase mass transport coefficient (hr^{-1}-m^{-2})
k_L	=	Liquid phase mass transport coefficient (hr^{-1}-m^{-2})
k_La	=	Volumetric mass transport coefficient (hr^{-1})
K_S	=	Michaelis constant (g/L)
$N_{A,L}$	=	Molar diffusion flux of oxygen into the liquid phase (mmole O_2/L-hr)
PA	=	Partial pressure of Oxygen (atm)
PCV	=	Packed cell volume
q	=	Specific respiration rate (mmole O2/g-hr)
Q_o	=	Volumetric respiration rate (mmole O2/L-hr)
So	=	Initial sucrose concentration (g/L)
τ_E	=	D.O. Electrode time constant

NOMENCLATURE AND ABBREVIATIONS (CONTINUED)

t_o = Initial time (hr)

μ = Average specific growth rate (day^{-1})

μ_{MAX} = Maximum specific growth rate (day^{-1})

V = Total volume of medium (L)

X = Biomass concentration (g/L)

X_{MAX} = Maximum biomass concentration (g/L)

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INTRODUCTION

Plant cell cultures have potential to produce high value products such as flavorings (vanilla and strawberry), fragrances (rose and sandalwood), and pharmaceuticals (codeine and digoxin). In fact, plant derived pharmaceuticals constitute one fourth of all prescriptions in the United States [Curtin, 1983]. These plant derived pharmaceuticals make up a nine billion dollar annual market [Kargi and Rosenberg,1987]. With the wide range of high value compounds produced by plants there is a great need for development of bioreactors suitable for large scale cultivation of plant cells. However, very few commercial operations exist for plant cell cultivation in bioreactors. The major problems impeding commercialization are: slow rates of production, shear sensitivity of plant cells, and low product yield [Kargi and Rosenberg,1987]. As a result, the product value must be in excess of $1000/kg in order to economically justify production on a large scale [Lyderson, 1987].

Bioreactor configurations and operating conditions are developed to provide more favorable conditions for the production of secondary metabolites at high cell density. Cell

concentration in a bioreactor is often limited by the capacity to supply sufficient oxygen to the cells. The limitation occurs because of insufficient gas-liquid mass transfer of oxygen. A way to determine the effects of a bioreactor's design and process conditions on the transfer of oxygen is by estimating the volumetric mass transfer coefficient, k_La. A method for *in situ* estimation of k_La during the cultivation period in a bioreactor has not been researched for plant cell culture.

Prior to implementing experimental methods for estimating k_La in a bioreactor, the cell culture must be characterized, and process parameters for optimum cultivation must be investigated. Cell cultures are characterized in shake flasks because of the highly favorable conditions for cell growth. Information obtained from the shake flask studies are then compared to bioreactor studies. Two different techniques may be employed to determine optimum process conditions for cell cultivation in a bioreactor. One method involves varying the process parameters one at a time until desired results are obtained. The other method involves measuring a range of initial k_La values on the medium in a bioreactor for various sets of process conditions. An acceptable range of initial k_La values are then determined that produce successful cell cultivation. After investigation of the process parameters and characterization of the cell culture, a model to estimate k_La during the cultivation period can be accessed.

Actual estimates of k_La would provide fundamental data on the effects of process variables on bioreactor performance. This information could be used for study of gas transport characteristics, or for design and scale-up calculations. Thus, the objectives of this research are to: (1) characterize cell suspension cultures in shake flasks of *Nicotiana tabacum* var. Wisconsin 38, (2) determine a set of conditions that result in acceptable cultivation of plant cells in a stirred-tank bioreactor, and (3) under these conditions, develop and evaluate an *in situ* method to estimate k_La as a function of time and cell concentration within a stirred-tank bioreactor during the cultivation period. The development of the methodology for *in situ* measurement of k_La would further advance technology of plant cell cultures.

LITERATURE REVIEW

Previous investigations covering both k_La estimates and plant cell cultivation are described below. Methodology already developed to estimate k_La in water and fermentation medium in a bioreactor are discused. The procedure most commonly used for k_La estimates in biological systems, the dynamic method, is thoroughly covered. Also reported are literature findings on both plant cell cultivation and mass transfer of oxygen in a stirred-tank reactor. The literature findings have then been incorporated into the development and evaluation of the method for *in situ* measurement of k_La in a stirred-tank bioreactor.

K_La Measurement

Various methods for estimation of the volumetric oxygen transfer coefficient (k_La) in bioreactors have been developed [Rainer, 1990]. For a biological system, a direct method is preferable. For cell culture studies, the direct method refers to measuring k_La in the presence of growing cells. For a batch fermentation process, a dynamic method is often used to measure the mass transfer coefficient k_La because it is accurate, convenient, and easy to implement. The technique, originally developed by Bandyopadhyay et al. [1967], measures the dissolved oxygen concentration in the liquid phase as a function of time after a short interruption in the inlet gas. Bandyopadhyay's model, the modified gassing out method,

considers only the liquid phase dynamics and assumes the gas and liquid phase in the vessel to be well mixed. However, if the vessel with a tank height versus tank diameter ratio is much less than 1, the well mixed assumption may prove faulty [Van't Riet, 1979] Also, if the gas phase residence time is much less than $1/k_La$, the oxygen concentration in the gas phase can no longer be regarded as a constant [Van't Riet, 1979].

Dang et. al. [1977] presented a revised dynamic method which accounts for both the gas and liquid film effects, along with the dissolved oxygen electrode dynamics. The method involves measuring k_La under cell-free conditions in a biorector. Dang developed four first order differential equations: gas phase oxygen balance, liquid phase oxygen balance, diffusion film lag, and membrane response for the dissolved oxygen (D.O.) electrode. The equations were combined to form a linear model with two parameters, k_La and the D.O. electrode time constant (τ_E). Two response curves of oxygen versus time are generated. First, an instantaneous change in dissolved oxygen was created by rapidly removing the probe from an oxygen free environment and submerging it into a saturated solution. Second a step change was created by stripping the solution with nitrogen and then re-aerating with air. The areas above the response curves (first moment) are related to the model parameters. From the instantaneous response curve τ_E is determined, and for the step response curve k_La can be estimated.

Ruchti et al. [1981] further investigated the model proposed by Dang [Dang, 1977]. Ruchti indicated that dynamic methods are usually quit sensitive to the starting conditions of the step change. The time variation during the period in which the nitrogen is shut off, and the air is turned on, can cause errors in k_La as high as 30%. Ruchti attributed the error to a hold up of nitrogen that is longer than the time necessary for k_La to become a constant after changing the gas inlet conditions. Ruchti proposed a new graphical method using $\log(1-C_E)$ vs time data where C_E is the normalized oxygen concentration in the reactor, to correct for variations of gas phase oxygen during gas interchange.

Linek et al. [1982,1985] investigated the validity of two different methods used to perform a step change in the dissolved oxygen level for the dynamic method. The first method (method A) consists of bringing about an oxygen step change in a batch process by switching gases while maintaining a constant volume gas flow rate and stirring speed. The second method (method B) used previously by Dang, involves first stripping the system of oxygen, then simultaneously starting up both the aeration and agitation of the liquid in a batch reactor. The dynamic model for method A assumes an ideally well mixed system which, according to Linek is erroneous because it fails to describe the mixing of gases during the gas interchange phase. Linek also indicates the model for method B is faulty because if an inert gas is used to bring the oxygen level down and oxygen to bring it

back up, the model fails to account for the added components transfering across the gas-liquid interface, and a low k_La value may result. Method B would be satisfactory if only a negligible change in oxygen concentration occurs in passing through the liquid. Linek accomplishes this by using a oxygen step change, and thus recommends this procedure for estimating k_La.

Chang et al. [1989] investigated the use of the dynamic method for microbial fermentation processes. Chang used oxygen enriched air for a gassing-in period and de-gassed with air to obtain the necessary step change to calculate k_La. The inhomogeneity in the hold up bubbles during the gas interruption stage was considerably less than for the previously used nitrogen/air gassing in-out process. The assumption of a well mixed system was also further validated. Chang also indicated that an uninterrupted air feed eliminates respiration rate calculations normally associated with fermentation systems. Two different methods were investigated to calculate k_La from experimental data: a slope method and a nonlinear least-squares regression. Chang claimed that the slope method was more reliable and less sensitive to electrode response times.

Bioreactor Cultivation of Plant Cells

To cultivate plant cells at high density, a suitable bioreactor must be found that offers optimum aeration and agitation conditions. Factors to consider are the ability of the reactor system to supply ample oxygen, sufficient culture broth

mixing, and air bubble dispersion while maintaining a low hydrodynamic stress [Tanaka, 1981]. Other important criteria for evaluating a bioreactor's overall performance are the ability to control environmental conditions, scale-up capabilities, and ease of operation.

Kargi and Rosenberg [1987] summarized and compared several reactor configurations developed for plant cell cultures. Discused were two different forms of reactors, those for suspension cultures and those using immobilized plant cells. Two types of suspension culture reactors were discussed, mechanically agitated reactors and nonmechanically agitated reactors. The types of immobilized cell reactors discussed were gel entrapped systems and membrane systems. On the basis of known characteristics of plant cells, Kargi felt that fluidized bed, airlift, or bubble column reactors with gel beads containing immobilized cells were the most appropriate reactor configurations. The reason cited is that gel entrapped cells promise a high degree of cell to cell contact and intercellular organization.

Another review by Panda et al. [1989] also considered various bioreactor configurations and their potential cultivations of plant cell suspensions for the production of secondary metabolites. Four types of cell suspension reactors were discussed: stirred-tank, bubble column, air-lift, and rotating drum. Panda et al. compare the different reactor types on the basis of oxygen transfer, hydrodynamic stress, mixing, and

scale-up. They conclude that stirred-tank reactors are most desirable for cell suspension cultivation of plant cells. Immobilized plant cell reactors were also discussed. Panda recommends an immobilized cell reactors for plant cell cultures in which growth and metabolic production are coupled, and stirred tank reactors when production of desired product is growth associated.

Payne et al. [1987], using a model system of Indole alkaloid production from *C. roseus*, investigated immobilized cell systems and a bubble column reactor. Based on this study, Payne reported a suppressed cellular metabolism when using immobilized cell reactors, and that the dynamics of growth and alkaloid production observed from preliminary shake flask studies could be reproduced in the bubble column.

Several successful plant cell cultivations have been performed using stirred-tank bioreactors. *Ammi visnaga* (L) Lam. was cultivated using a 3.5 L stirred-tank reactor and reached a maximum dry cell yield of 1.083 g/L/day [Kaul and Staba, 1972]. *Medicago sativa* L. was successfully cultivated in a 500 mL stirred-tank reactor using a marine blade impeller. The maximum cell density obtained was about 8.9 g dry weight/L [McDonald and Jackman, 1989]. Kato et al. [1971] cultivated tobacco cells in a stirred-tank reactor using two flat blade turbine disc impellers. The final dry cell density of the tobacco cells in the reactor was 20 g/L. Several successful cultivations of *C. roseus* cells have been reported using different types of

impellers and agitation rates. Drapeau et al. [1985] cultivated *C. roseus* cells using a 10 L vessel and a flat blade turbine impeller. Different agitation rates were used ranging from 80 to 100 rpm. A maximum dry cell density of around 8 g/L was obtained. Ducos et al. [1988] also employed a 10 L vessel, using a double pitched blade turbine impeller at an agitation rate of 80 rpm. The maximum dry cell density was about 22 g/L. Leckie et al. [1990] cultivated *C. roseus* in a 8.5 L vessel using a 6-blade Ruston impeller and varied the agitation rates from 100 to 300 rpm. A maximum wet cell density of about 200 g/L was obtained.

As can be assessed from above, different impeller designs and agitation speeds affect cultivation. Hooker et al. [1990] investigated the effects of impeller design and agitation speeds on the growth rate and cell density of tobacco cells in a 5 L jar fermenter. An ideal mixing speed of 150 rpm (tip speed equalling 43.9 cm/sec.) was found for a flat blade impeller producing a maximum wet cell density of 520 g/L for tobacco cells. A paddle impeller was reported to be preferable to a flat blade. In addition, as the blade width increased, cell growth also increased for the paddle impeller. For a blade width of 5.1 cm, maximum wet cell density was about 295 g/L, and for 14 cm, 580 g/L. A new sail impeller was tested and resulted in an increased maximum growth rate but a longer lag phase than the paddle blade impellers.

Aeration and agitation affects the shear environment of the cells. Hooker et al. [1989] investigated the effects of

hydrodynamic shear stress on plant cells. Their study indicated cell suspension cultures in the lag phase and later stationary phase are less susceptible to shear damage than cells in latter stages of exponential growth phase and early stationary phase.

In addition to process parameters for a stirred-tank reactor, sugar levels in the medium have been investigated with the aim of increasing cell density. Reports of an increase in cell mass for an increase in sugar concentration for *Discorea deltoidea* and for *C. roseus* have been reported by Drapeau et al. [1985]. Their study correlated the decrease in batch growth rate, and the ratio of dry to fresh weight to the decline in sugar concentration in the later half of the growth phase for both *D. deltoidea* and *C. roseus*. Schiel and Berlin [1987] also reported differences in dry to fresh cell weights during cultivation of *C. roseus* in a fermenter. A possible reason given by Drapeau for the differences in fresh and dry cell weights is the osmotic effect related to sucrose levels. If the osmotic potential governs the degree of cell expansion, altering the medium to decrease cell size may lessen the sensitivity of plant cells to hydrodynamic stress.

Oxygen Mass Transfer in Plant Cell Cultures

The growth of plant cell cultures is greatly influenced by oxygen supply. Snape et al. [1989] investigated the effects of oxygen supply on the growth of *C. roseus* in 100 mL Erlenmeyer

shake flasks. They found the oxygen demand of the culture depends on the stage of growth cycle of the cells. Also, a critical oxygen level was observed where cultures could not survive below the critical dissolved oxygen concentration. A study by Kessell and Carr [1972] reported similar findings concerning the effect of dissolved oxygen concentration on the growth of *Daucus carota* using a stirred-tank reactor. A critical oxygen level was observed in this study where cells grew at a constant rate below the critical dissolved oxygen level, and grew exponentially above the critical level. Also observed where changes in the nutrient uptake during the period when dissolved oxygen was below the critical level, thus indicating changes in cell metabolism.

The level of dissolved oxygen in a stirred-tank reactor is related to the aeration rate in the vessel. Yamakawa et al. 1982] investigated the effects of aeration rate on *Vitis* cells and anthocyanin formation. Using a 2 L fermenter, the aeration rate was varied from 0.2 to 0.8 vvm. The highest yield of both cell mass and anthocyanin were attained at an optimal aeration rate of o.4 vvm. Yamakawa also investigated the effects aeration rate had on initial k_La values. A modified gassing out technique was used to measure initial k_La values, and a linear relationship was reported to exist between the two parameters.

The mass transfer coefficient not only depends on aeration rates, but other properties in a bioreactor system. Tanaka [1981] performed experiments with shake flasks to determine k_La as a

function of plant cell growth. Respiration rates of samples withdrawn from a shake flask were determined by a previously developed method [Takahashi and Yoshida, 1979]. The model used to calculate k_La includes the respiration rate in the global oxygen balance and assumes both a quasi-steady state in the flask and a direct linear proportionality between the dissolved oxygen level in the medium and the dissolved oxygen probe reading. The results showed that k_La increased with decreasing cell mass.

Studies have been performed using initial k_La values to determine optimum bioreactor designs. Tanaka [1981], using a model of water/ ß-naphthol particles to simulate a plant cell culture, investigated oxygen mass transfer in different bioreactor systems. The studies consisted of measuring K (the solid-liquid mass transfer coefficient) for the ß-naphthol system in various bioreactors and using it as an index to measure the intensity of hydrodynamic stress. The intensity of culture broth mixing and air bubble dispersion was gauged by measuring the initial k_La, using a modified gassing out method, on the different bioreactors with plant cells present. The two indexes, K and k_La, were then compared with cell mass concentrations in the reactors. A K value of less than 0.0044 cm/sec was obtained for normal cell growth. Based on these studies a jar fermenter with a paddle type impeller was concluded to be best suited to cultivate plant cells at high density.

Hashimoto and Azechi [1988] measured k_La as a function of culture growth in a batch fermenter. The results showed k_La reached a maximum value and then decreased over the culture period. The liquid volume and method of measurement for k_La were not reported.

Kato et al. [1975] investigated the effects of aeration and agitation conditions on the initial k_La for batch cultivations of tobacco cells. Initial k_La values for different sets of aeration and agitation conditions were determined in water using the modified gassing out method. They found that k_La did not affect the lag phase of cell growth. However, as the culture time increased, biomass production increased linearly with increasing k_La for $k_La \leq 10$ hr^{-1}. The biomass production was constant for $k_La > 10$ hr^{-1}.

A similar study by Leckie et al. [1990] investigated the effects of initial k_La on cell growth rates of *Catharanthus roseus*. The initial k_La value was altered from 2.5 to 20 hr^{-1} by increasing the aeration rate and changing the air sparger design. Again, the initial k_La was measured in water using the modified gassing out method. Results showed an optimum k_La range of 4.5 to 12.5 hr^{-1} for maximum biomass production. Oxygen uptake rates were measured on samples withdrawn from the bioreactor. The maximum uptake rate, 0.0052 mmol O_2/g dry wt-hr, occurred at an initial k_La value of 20 hr^{-1} and was shown to be about five times higher than the oxygen uptake rate obtained from shake flask cultures.

Variations in oxygen and carbon dioxide aeration rates were studied [Ducos and Parelleux, 1986] using *C. roseus* cells in a bioreactor. When the aeration rate was increased from 0.4 to 1.5 vvm, the global growth rate and conversion yield (g of cells minus g of initial cells / g of initial sucrose minus g of sugar) decreased. In a parallel experiment, the carbon dioxide partial pressure in the medium was maintained at 20 mbar. No detrimental effects on growth rate and conversion yields were observed when the aeration rate was increased. Thus, neither hydrodynamic stress or dissolved oxygen tension were responsible for the decrease in the global growth rate of the cells. Similar studies showing the beneficial effects of carbon dioxide on cellular growth rate were performed with *C. roseus* using an air-lift bioreactor [Hegarty et al., 1986].

The growth promoting effect of CO_2 was further investigated by Ducos et al. [1988]. The enzyme activities of glycolysis, pentose-phosphate during a batch cultivation of *C. roseus* was monitored. They found enzyme activities were dependent on the culture gas environment involving the pressure of CO_2 during early stages of growth. Thus dissolved carbon dioxide acted as a 'conditioning factor' on the culture medium and has been recommended to be considered as an essential process variable for the optimization growth of plant cells in large scale bioreactors.

The maintenance of oxygen and carbon dioxide levels in a stirred-tank reactor was investigated by Smith et al. [1989].

They developed a control algorithm that is reported to maintain a set point level for both dissolved oxygen level and carbon dioxide partial pressure in a stirred tank reactor. The control algorithm originally used k_La as the only parameter. However, after measuring k_La (actual experimental procedures were not discussed) during the cultivation of *C. roseus* using a stirred-tank reactor, k_La was found to be relatively constant and thus independent of the cell concentration. Their control method was designed on the assumption that k_La was constant, and that the respiration rates could be used as a control parameter. Respiration rates were measured assuming that: (1) steady state gas concentration existed in the reactor during the time of measurement, (2) the inlet gas concentration was constant, and (3) the set point for the gas reflected the actual gas concentration in the reactor. Smith reported success in maintaining the set point levels for both dissolved oxygen and carbon dioxide concentrations in the cultivation of *C. roseus*.

MODEL DEVELOPMENT AND DATA ANALYSIS

When the medium in the stirred-tank bioreactor is aerated with air, air bubbles rise through the medium, and oxygen is transfered from the gas phase to the liquid medium. The transfer of oxygen from the gas phase to the liquid phase is governed by two film theory [Rainer, 1990]. According to two-film theory, the molar diffusion flux of oxygen into the liquid phase ($N_{A,L}$) can be represented by:

$$N_{A,L} = K_L(C_A^* - C_{A,L}) \tag{1}$$

where the subscript "A" indicates oxygen, K_L is the overall liquid phase mass transfer coefficient, C_A^* is the concentration of dissolved oxygen in equilibrium with the partial pressure oxygen in the gas phase, and $C_{A,L}$ is the bulk dissolved oxygen concentration.

The equilibrium distribution of oxygen between the gas and liquid phases in the reactor can be expressed by Henry's law. Henry's law relates the term C_A^* to the partial pressure of oxygen (P_A)

$$P_A = H\, C_A^* \tag{2}$$

where H is the Henry's law constant. Oxygen is a relatively insoluble in water, and thus H is large. As a result, the major

resistance to oxygen mass transfer is in the liquid phase, and K_L can be approximated by k_L, the liquid phase mass transfer coefficient. Equation (1) simplifies to:

$$N_{A,L} = k_L(C_A^* - C_{A,L}) \tag{3}$$

The development of a model to describe the unsteady state oxygen concentration in an aerated stirred-tank reactor requires some simplifying assumptions. The liquid volume and temperature are assumed constant throughout the cultivation period. Also, an ideally well mixed gas phase in the medium is assumed. If these assumptions are incorporated into the global oxygen balance for the bioreactor, the resulting unsteady state oxygen concentration in the liquid phase can be represented by:

$$\frac{dC_{A,L}}{dt} = k\frac{A}{V}(C_A^* - C_{A,L}) \tag{4}$$

where A is the total surface area of the air bubbles available for interphase mass transfer of oxygen, and V is the total volume of medium. The surface area of the air bubbles is virtually impossible to measure. For this reason the factor "a" is introduced to represent the interfacial surface area for oxygen mass transfer per unit volume of the medium in the bioreactor. Equation (4) then becomes

$$\frac{dC_{A,L}}{dt} = k_L a(C_A^* - C_{A,L}) \qquad (5)$$

where $k_L a$ is the volumetric oxygen mass transfer coefficient.

When aerobic plant cells are in the reactor, they consume dissolved oxygen by the process of respiration. Therefore, for an actively growing culture, the volumetric respiration rate (Q_o) must be included in the liquid phase oxygen balance:

$$\frac{dC_{A,L}}{dt} = k_L a(C_A^* - C_{A,L}) - Q_o \qquad (6)$$

The volumetric respiration rate is the product of the specific oxygen uptake rate (q_o) and the biomass concentration (X(t)):

$$Q_o = q_o X(t) \qquad (7)$$

When equation (7) is inserted into equation (6), the differential equation which describes the unsteady state liquid phase oxygen concentration in the bioreactor is:

$$\frac{dC_{A,L}}{dt} = k_L a(C_A^* - C_{A,L}) - q_o X(t) \qquad (8)$$

Equation (8) is subject to the initial conditions $t = t_o$ and $C_{A,L} = C_{A,o}$. If the air supply to the bioreactor is switched off, the diffusion flux of oxygen into the liquid becomes zero, and the oxygen concentration decreases according to the oxygen consumption of the cells. The biomass concentration is assumed to be constant during the short period in which the air is shut off, and thus is independent of cultivation time. As a result, during the non-aerating phase equation (8) reduces to

$$\frac{dC_A}{dt} = -q_o X \tag{9}$$

which when integrated, yields

$$C_A = C_{A,o} - q_o X(t - t_o) \tag{10}$$

where t_o and $C_{A,o}$ are the time and dissolved oxygen concentration at the point when the air is turned off. From equation (10), a linear dependence of C_A on t with a slope of $-q_o X$ is predicted.

Parameter Evaluation

The evaluation of $k_L a$ requires two step changes on the aerating gas. In the first step change, the air flow to the reactor is shut off. If cells are present in the reactor, the oxygen concentration will decrease linearly according to equation (10). The slope of the C_A vs t curve produced by the first step change (section I in Figure 1) is the volumetric respiration rate (Q_o).

When the oxygen concentration drops 30% to 50% from the initial steady state value, but still above the critical oxygen level (0.0127 mmole O_2/L [Lyderson, 1987]), the air is turned back on to initiate the second step change. The oxygen concentration rises and eventually reaches a steady state value. Section II in Figure 1 represents the second step change. The C_A vs t data obtained during the re-aerating step is used to estimate k_La by a non-linear regression technique.

The Gauss-Newton nonlinear regression analysis is employed to determine k_La from the C_A vs t curve produced during the re-aeration step. The method involves fitting nonlinear equation (10) by a least squares iterative method. The algorithm used to predict k_La by the Gauss-Newton method is

$$k_La^{m+1} = k_La^{m} + \frac{\sum_{i=1}^{n} \frac{dC_A(t_i)}{dk_La} (C_{A,i}-C_A(t_i))}{\sum_{i=1}^{n} \left(\frac{dC_A(t_i)}{dk_La}\right)^2} \qquad (11)$$

where $C_A(t_i)$ is the predicted dissolved oxygen concentration at time t_i for the current (m^{th}) estimate of k_La, $C_{A,i}$ is the measured

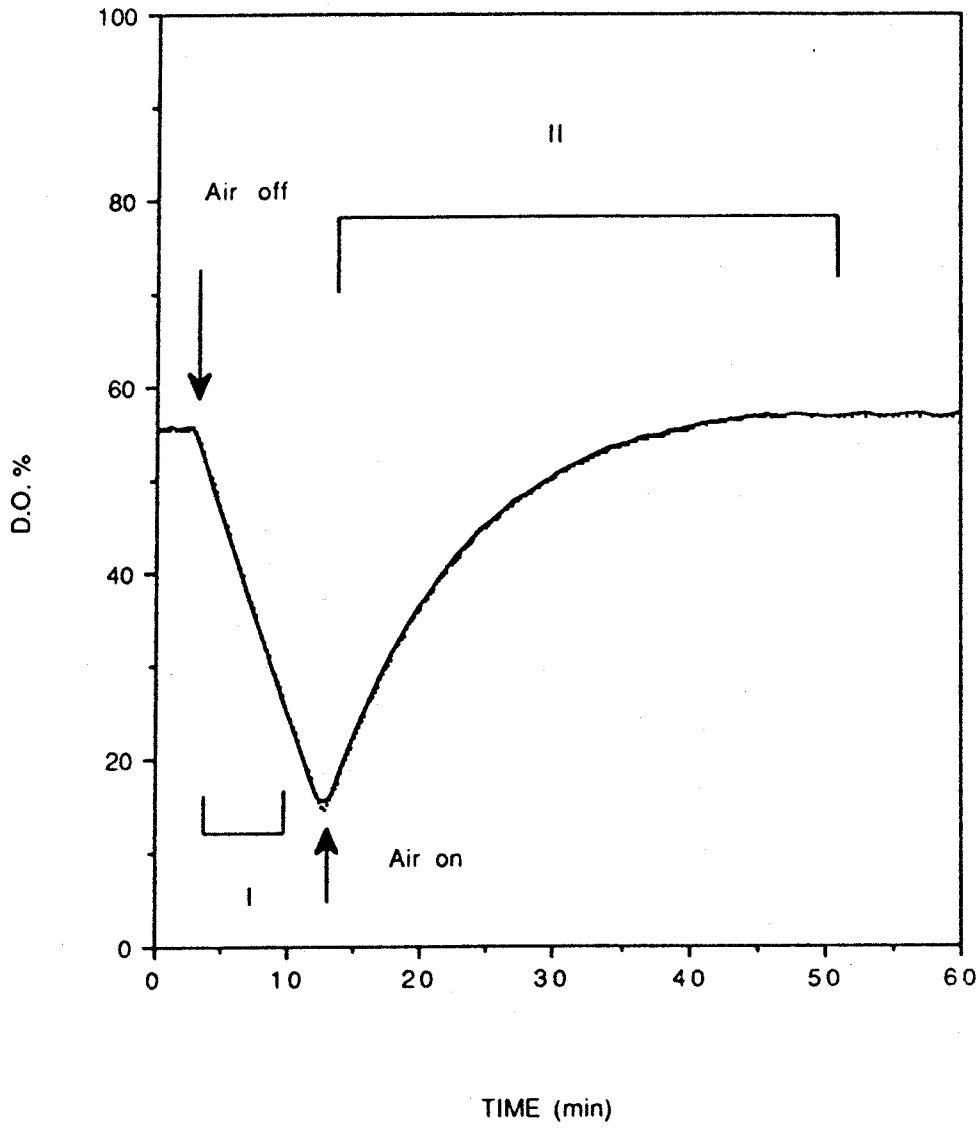

Figure 1. Step changes in aeration for cell cultivation in a 2L stirred-tank bioreactor. The slope in section I represents the volumetric respiration rate (Q_o). The data in section II is used to calculate k_La by a nonlinear regression technique.

dissolved oxygen concentration at time t_i, and n is the number of data points.

If it is assumed that the volumetric respiration rate (Q_o) remains a constant during the re-aeration step, then the solution of the ordinary differential equation (8) is

$$C_{A,L} = C_{A,L}^* - q_o X/k_L a - [C_{A,L}^* - q_o X/k_L a - C_{A,o}]\exp(-k_L a(t-t_o)) \qquad (12)$$

The "sensitivity" equation, given by the derivative of equation (12) with respect the estimated parameter ($k_L a$) is

$$\frac{dC_A}{dk_L a} = \frac{q_o X}{(k_L a)^2}[1-\exp(-k_L a(t-t_o)] - [C_{A,o} - C_A^* + q_o X/k_L a](t-t_o)\exp(-k_L a(t-t_o)) \qquad (13)$$

Equations (12) and (13) are then used in equation (11). A computer program given in Appendix A estimated Q_o and $k_L a$ from C_A vs t pulse test data. Specifically, Q_o was estimated by linear regression of C_A vs t data during the non-gassing phase (air off), and $k_L a$ was estimated by nonlinear regression of C_A vs t data during the re-aeration phase. The nonlinear regression estimation required an initial guess for $k_L a$. The $k_L a$ for the medium alone (7.72 hr^{-1}) was used as the initial guess.

The initial value of k_La for the medium alone at a fixed set of process conditions was obtained by implementing a step change on the oxygen concentration on the medium in the reactor with no cells present. The step change was performed by de-gassing the medium with nitrogen, and then re-aerating the medium with air. The C_A vs t curve produced during the re-aerating phase enabled an initial k_La to be estimated using the nonlinear regression method discussed above by setting Q_o equal to zero. A value of 4 hr^{-1} was used as the initial guess for the nonlinear regression calculation of k_La.

Several assumptions were required for k_La estimation. When the initial k_La was estimated, the gas phase concentration of oxygen was considered constant. When the gas line was switched from nitrogen to air, the gas hold up of nitrogen during the interchange was considered to be insignificant. The time constant for the probe response was assumed to be negligible. During the period of respiration rate and k_La estimation, both the biomass concentration (X) and k_La were considered to be constant through out the vessel.

MATERIALS AND METHODS

SHAKE FLASK STUDIES

Culture Initiation and Maintenance

Three week old callus cultures of *Nicotiana tabacum* var. Wisconsin 38 (low cytokinin dependent cell line) were obtained from Dr. Donald Armstrong, Department of Botany, Oregon State University. The medium composition for the callus cultures can be found in Table 1. Callus culture were kept in a darkened cabinet at 28 °C and subcultured every three weeks. To subculture the callus, flame sterilized forceps were used to remove the callus cultures from the old flask into a sterilized petri dish. In the dish, three loose, small white (friable) callus portions, about 2mm in diameter, were excised from the callus using a flame sterilized knife. The friable callus portions were then evenly distributed with flame sterilized forceps onto 30 mL of solidified medium in a 125 mL Erlenmeyer flask. All work was carried out in a laminar flow hood.

Cell suspension cultures were originally inoculated with friable portions of callus in a laminar flow hood. About 2.0 g of the friable callus portions were suspended in 30 mL of modified MS medium in a 125 mL Erlenmeyer flask using flame sterilized forceps. The modified MS medium composition can be found in Table 2. The media for both the callus and cell suspension cultures was autoclaved at 250 °F, 15 psig for 30 minutes. The pH of the medium was adjusted to 5.8-6.0 prior to autoclaving.

Table 1. Callus culture media composition.

Compound	Amount
MS Nutrient base	4.4 g/L
Ammonium Nitrate	1.65 g/L
Boric Acid	6.2 mg/L
Calcium Chloride (Anhydrous)	332.2 mg/L
Cobalt Chloride-6H_2O	0.025 mg/L
Cupric Sulfate-5H_2O	0.025 mg/L
Ethylenediamineteraacetic Acid	37.26 mg/L
Ferrous Sulfate-7H_2O	27.8 mg/L
Magnesium Sulfate	180.0 mg/L
Manganese Sulfate	16.9 mg/L
Molybolic Acid -2H_2O	0.25 mg/L
Potassium Iodide	0.83 g/L
Potassium Nitrate	1.9 g/L
Potassium Phosphate Monobasic	170.0 mg/L
Zinc Sulfate-7H_2O	8.6 mg/L
Glycine (free base)	2.0 mg/L
myo-Inositol	100.0 mg/L
Nicotinic Acid (free acid)	0.5 mg/L
Pyridoxine-HCl	0.5 mg/L
Thiamine-HCL	0.1 mg/L
IAA (Indole-3-Acetic Acid)	0.05 mg/L
Agar	8.0 g/L
Sucrose	30 g/L

Table 2. Suspension culture media composition.

Compound	Amount
MS Nutrient base	4.4 g/L
Ammonium Nitrate	1.65 g/L
Boric Acid	6.2 mg/L
Calcium Chloride (Anhydrous)	332.2 mg/L
Cobalt Chloride-6H_2O	0.025 mg/L
Cupric Sulfate-5H_2O	0.025 mg/L
Ethylenediamineteraacetic Acid	37.26 mg/L
Ferrous Sulfate-7H_2O	27.8 mg/L
Magnesium Sulfate	180.0 mg/L
Manganese Sulfate	16.9 mg/L
Molybolic Acid -2H_2O	0.25 mg/L
Potassium Iodide	0.83 g/L
Potassium Nitrate	1.9 g/L
Potassium Phosphate Monobasic	170.0 mg/L
Zinc Sulfate-7H_2O	8.6 mg/L
Glycine (free base)	2.0 mg/L
myo-Inositol	100.0 mg/L
Nicotinic Acid (free acid)	0.5 mg/L
Pyridoxine-HCl	0.5 mg/L
Thiamine-HCL	0.1 mg/L
2,4 - D (2,4-Dichlorophenoxyacetic Acid)	0.5 g/L
Kinetin (6-Furfurylaminopurine)	30 µg/L
Sucrose	30 g/L

The suspension cultures of *N. tabacum* were maintained in 125, 250, and 500 mL Erlenmeyer flasks by incubating in a Lab-line orbital environmental shaker at 27 °C. The conditions for maintaining the cultures are found in Table 3. Subculturing was performed approximately ten days with a 25 to 30% (v/v) inoculum. Specific inoculum volumes for each different flask size can be found in Table 4. A 10 mL (tip diameter = 1/8") autoclaved, flame sterilized pipet was used to transfer the inoculum into the fresh medium. The inoculation of a 500 mL flask containing 100 mL of fresh medium from a 125 mL flask containing 40 mL of suspended cells consisted of flaming both flask ends, pouring the cells from the 125 mL flask to the 500 mL flask, re-flaming the 500 mL flask, and then sealing it with a cotton plug wrapped and tied in cheeze cloth.

Cell Concentration Measurements

Cell concentration measurements were measured as both dry cell density and packed cell volume. Four 10 mL aliquots from two cell suspension flasks were poured into 15 mL conical centrifuge tubes. Samples were centrifuged for ten minutes at 2000 rpm (523 g) on a IEC Centra 4B centrifuge fitted with an IEC 320 rotor. The supernate was withdrawn with a pasteur pipet and measured in a 10 mL volumetric cylinder. The packed-cell volume (PCV, volume of cells/culture volume) was determined by the difference of the supernate and the total culture volume in the tube. The centrifuged cell mass was

Table 3. Shake flask culture conditions.

Flask Size (mL) Volume	Shake Speed (RPM)	Total Liquid
125	150	40
250	130	70
500	115	140

Note: Orbital shaker displacement of 3/4", and temperature at 27 °C.

Table 4. Media inoculation volumes.

Flask Size (mL)	Medium (mL)	Inoculum (mL)	(v/v)
125	30	10	25
250	50	20	29
500	100	40	29

resuspended in distilled water, filtered through pre-weighed Whatman filter paper #1 (55 mm diameter), and then dried at 60 °C for 48 hours. The dried filter paper was weighed, and the dry cell density (g dry cells/L) determined by the difference between the filter paper with and without cells divided by the original 10 mL of sample.

Conductivity Measurements

Conductivity measurements were made on the decanted culture supernate at 21 °C using a Markson ElectroMark conductivity meter. The supernate was placed in Fisher brand glass vials (size 2DR, 7.5 mL), and the conductivity probe submerged to a length of 2 cm. The conductivity probe was calibrated with molar solutions (0.2 to 1.747 M) of potassium nitrate at 21 °C.

Sugar Concentration Measurements

Sugar concentrations in the culture liquid were measured by high-performance liquid chromatography (HPLC). A Waters 501 isocratic pump, Rheodyne 725 injector, Eldex column oven, Altex model 147 refractive index detector, and a Beckman 427 integrator comprised the HPLC system. Table 5 summarizes the analysis conditions. Concentrations of sucrose, glucose, and fructose were calculated from peak area data by the internal standard method, using sorbitol as the internal standard. The

Table 5. Column and operating conditions for HPLC.

Parameter	Setting and Units
Column	BIORAD HPX- 87P
Solvent	HPLC-grade, He-degassed water
Flow Rate	0.6 mL/min
Column Temperature	85 °C
RI Detector Range	4x
Back Pressure	600 psig

retention times of sucrose, glucose, fructose, and sorbitol were 10.4, 12.6, 16.7 and 35.7 minutes respectively.

Prior to HPLC analysis, 1 mL of the liquid culture was mixed with 1 mL of 30 g/L of sorbitol and filtered through a 0.45 µm filter. A 20 µL sample was injected into the column.

BIOREACTOR STUDIES

Bioreactor Description

A VirTis 2 L stirred-tank fermenter with a working volume of 1.5 L (14.5 cm in height and 10.5 cm in width) was used for the tobacco cell culture studies. Figure 2 presents a schematic of the bioreactor and Table 6 list the process and culture conditions for the bioreactor.

The culture was mixed with a paddle impeller of width equal to 1.7 cm and height equal to 4.5 cm. The base of the impeller was located 4.0 cm from the base of the vessel. Turbine and marine impellers were used for preliminary studies, and their dimensions can be found in Table 7. Cultures were sparged with humidified air at 0.7 L/min (.47 vvm) metered at 23 °C. The sparger consisted of one row of five 1.0 mm diameter holes. Nitrogen, when used as a blanket over the medium, was introduced into the bioreactor through a 0.2 µM diameter venting filter at 1.14 L/min. The bioreactor headplate was equipped with a VirTis galvanic oxygen electrode (D.O.), cartridge heater, RTD temperature probe, and three ports for venting with

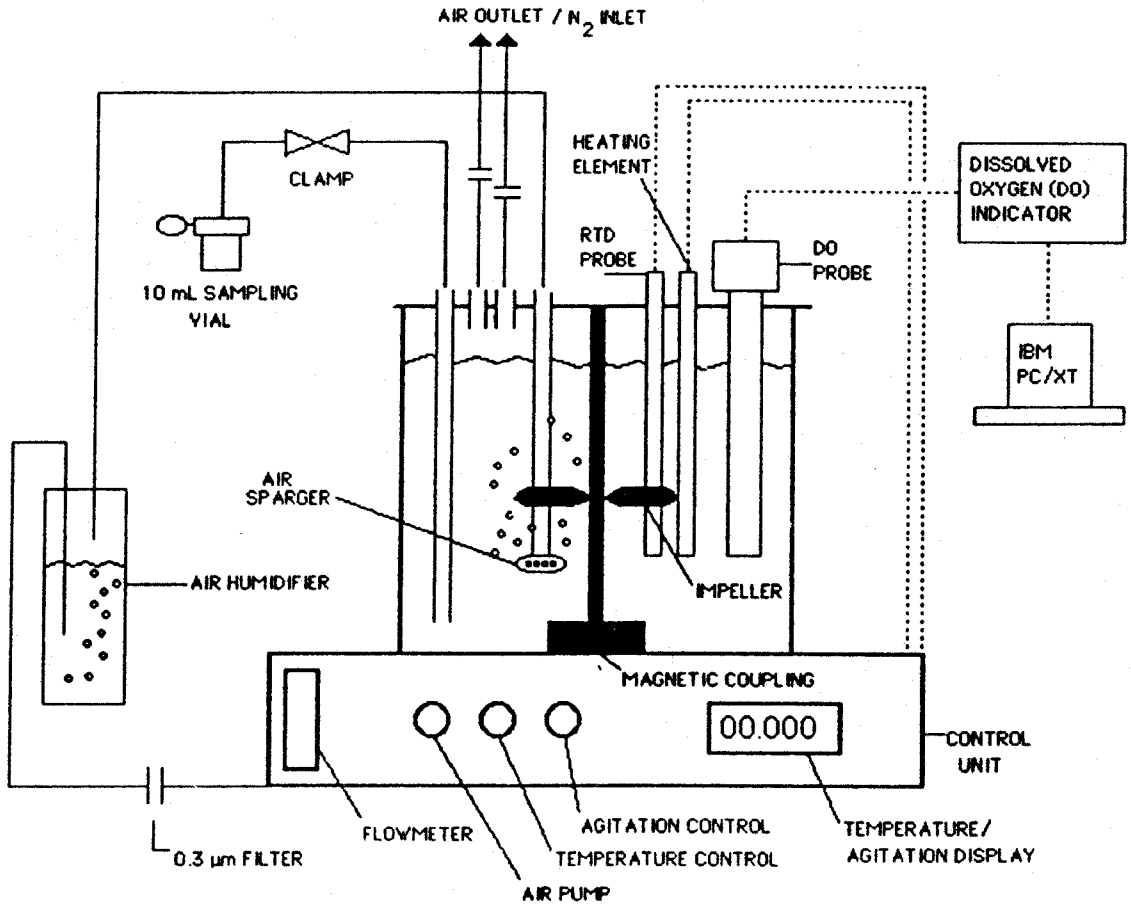

Figure 2. Two-liter stirred-tank bioreactor.

TABLE 6. Bioreactor process and culture conditions.

Process Conditions	Settings		
	Run 1	Run 2	Run 3
Air Flow Rate	0.7 LPM	0.7 LPM	0.8 LPM
Agitation Speed	150 rpm	150 rpm	150 rpm
Total Volume	1600 mL	1600 mL	1800mL
Inoculum Volume	300 mL	300 mL	500 mL
Inoculum Age	8 days	8 days	8 days
Inoculum Density	10 g/L	10 g/L	10 g/L
Temperature	27 °C	27 °C	27 °C
Initial Sucrose Concentration	30 g/L	30 g/L	42 g/L

Table 7. Dimensions for the impellers used in the bioreactor.

Impeller	Height (cm)	Diameter (cm)
Turbine	0.9	5.0
Marine	2.0	4.0
Paddle	4.5	5.5

0.2 μM filters. Culture samples were withdrawn through a 1/4" OD dip tube into a 20 mL autoclaved glass vial. The bioreactor was placed in a laminar flow hood to minimize contamination.

Bioreactor Start-up

The medium composition for the bioreactor was identical to that used for the shake flasks studies. The initial sucrose concentration in the medium was fixed at 30 g/L.

Prior to start-up and inoculation, the bioreactor was cleaned and sterilized. The 2 L glass vessel, headplate and attachments were scrubbed thoroughly with soap and water, soaked with 20% Clorox for 10 hours, and rinsed again 3 times with distilled water. The assembled headplate and vessel were then autocalved at 250 °F, 15 psig for one hour. Twelve hundred mL of medium was loaded into the vessel and the headplate resealed. The sealed bioreactor was placed in a autoclavable pan, sealed with a paper bag, and then autoclaved for 50 minutes. The bioreactor was then mounted onto the control unit and allowed to cool to 27 °C. After cooling, humidified air was sparged into the medium for 2 hours until it was fully saturated with dissolved oxygen. The D.O. electrode was electronically zeroed and then calibrated by setting air-saturated medium equal to 100%.

Initial k_La measurements were taken on the bioreactor with only medium present. Nitrogen was sparged into the medium through the air sparger to strip the medium of dissolved

oxygen. After stripping, nitrogen was introduced through one of the venting filters to blanket the medium. Humidified air was sparged into the bioreactor to re-aerate the medium. The dissolved oxygen concentration was monitored as a function of time by a VirTis dissolved oxygen meter. The analog output from the meter was sent to a computer data acquisition comprised of a Data Translation DT2801 I/O Board and IBM PC/XT computer. A BASIC program using PCLAB subroutines processed and stored the incoming concentration versus time data and plotted out the % D.O. in real time to the CRT display. The program listing is given in Appendix A. The change in steady state dissolved oxygen concentration when nitrogen was and was not blanketed over the medium was also measured before inoculating the bioreactor.

The bioreactor was inoculated with three 500 mL flasks each containing 140 mL of 11 day old tobacco suspension cultures. The D.O. electrode was removed from the vessel, and an autoclaved and flame-sterilized funnel inserted into the port. The three flasks were then individually flamed at the tip and poured into the reactor through the funnel. The inoculum did not significantly adhere to the sides of the glass. Once the inoculum was completely added, the electrode was resealed into the headplate.

Bioreactor Operation

Daily measurements of Q_o and k_La values, microscopic cell counts, pH, conductivity, and sucrose utilization were performed

in triplicate. Prior to the oxygen mass transfer experiments, nitrogen was sparged over the medium at 1.14 L/min. The dissolved oxygen concentration was allow to reach a new steady state value. The oxygen mass transfer experiments involve shutting off the air supply until the dissolve oxygen concentration decreased 30% from the initial steady state value (% D.O. not allowed to drop below 5%). The culture was then re-aerating until steady state was again reached. The oxygen concentration versus time profiles were monitored by the VirTis D.O. meter interfaced to the PC data acquisition system. After completing the measurements, a 20 mL sample was withdrawn from the reactor and a 100 μL sample was inspected under the microscope. A Fuchs Rosenthal hemocytometer was used to determine cell, cell fraction, and agglomerate counts. The rest of the sample was used to measure: pH, PCV, dry cell density, conductivity, and sugar utilization as described earlier.

D.O. Electrode Time Constant

The D.O. electrode response time was measured assuming a first order relationship for the response time delay [Ho Nam et al., 1989].

$$\frac{dC_{A,E}}{dt} = \frac{C_{A,E} - C_{O_2,L}}{\tau_E} \qquad (14)$$

where $C_{A,E}$ is the calibrated D.O. electrode reading for the oxygen concentration. The electrode was initially submerged in an oxygen free sodium sulfite solution (25 g/L) and calibrated to zero percent. The electrode was then rapidly immersed into air-saturated medium in the bioreactor. The assumed first order response to the change in environment of the D.O. electrode was monitored on the computer and a electrode time constant (τ_E) determined from equation (14).

Microscopic Analysis

A Spencer bench top microscope with a 10X and a 43X lense were used to visually inspect the cell cultures. A pasteur pipet was used to place a drop onto a plain microscope slide and a Fuchs Rosenthal Ultra Plane Hemocytometer (1/16 mm^2 per square and 2/10 mm deep). The sample on the slide was first inspected for contamination. The hemocytometer was then used to make cell, cell fragment, and cell aggregate counts. The entire area of the hemocytometer was used to obtain the counts (20 mm^2). The number of individual cells, fragments, and aggregates per volume was then found by dividing the total counts of each by the total volume of the hemocytometer ($4 \cdot 10^{-03}$ mL).

RESULTS AND DISCUSSION

Cell cultures of *N. tabacum* var. Wisconsin 38 were characterized in shake flasks. Results from shake flask experiments are compared to previous literature studies on different varieties of tobacco cells. Cell cultivation of *N. tabacum* var. Wisconsin 38 in a stirred-tank bioreactor was also investigated. Bioreactor measurements focused on obtaining the volumetric respiration rate (Q_o) and mass transfer coefficient (k_La) as a function of culture time and cell density (X).

SHAKE FLASK STUDIES

Cell suspension tobacco cultures were characterized in shake flasks in a low shear environment. Important measurements included biomass growth rate, maximum cell density versus initial sucrose concentration (yield coefficient), and sugar utilization.

Growth Curve Measurements

The cell density in a 125 mL shake flask (27 ^oC, 150 rpm) was measured as a function of culture time (growth curve). Three different techniques for measuring cell biomass were employed: dry cell density, packed cell volume (PCV), and conductivity. Figure 3 shows the growth curve based on dry cell density for three replica experiments. The culture experiments

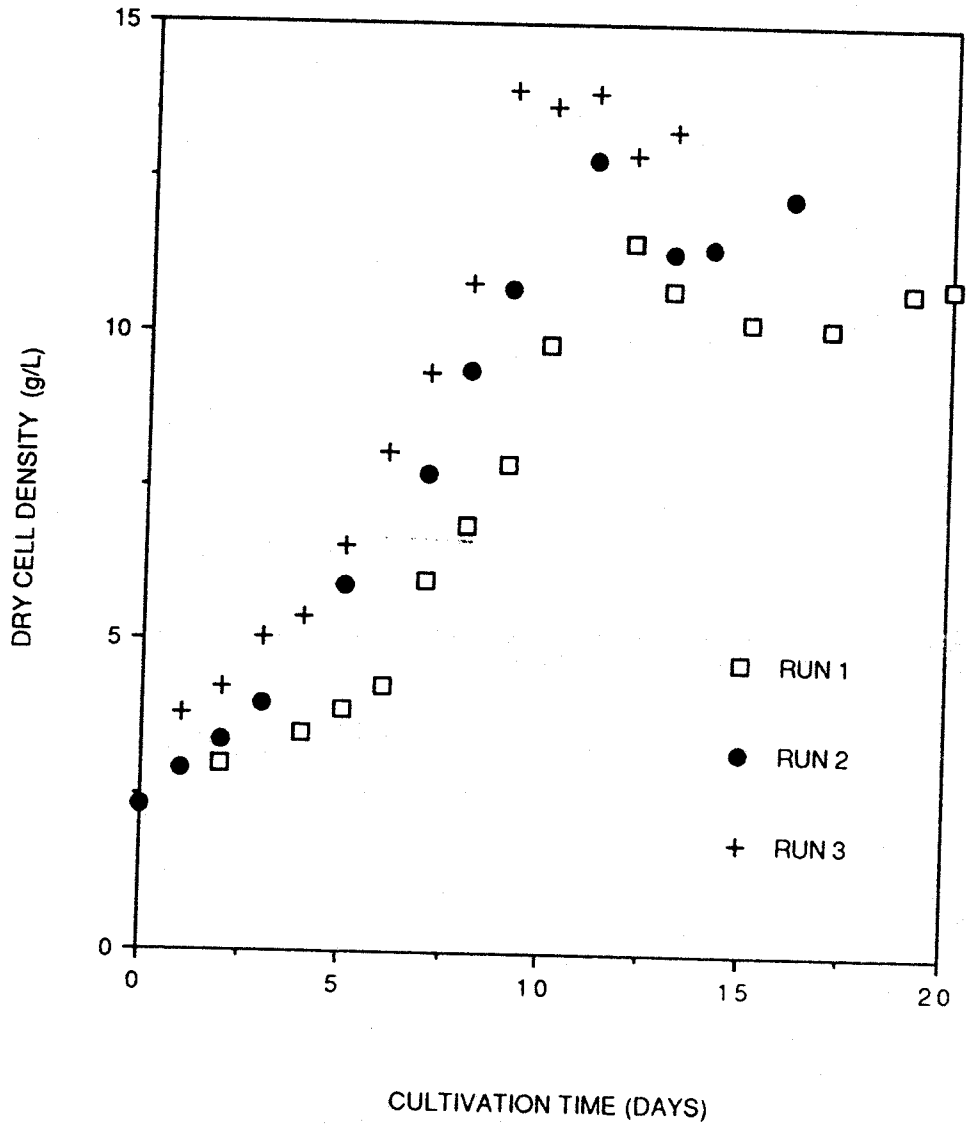

Figure 3. Repeatability of shake flask growth curve based on dry cell density. Conditions: 27 °C, 150 rpm, and 40 mL culture/ 125 mL flask.

typically had a lag phase of one day, and exponential phase of four to six days, and reached maximum cell density after ten days At the conditions given in Figure 4, an average specific growth rate (μ) of 0.070 ± 0.005 g/L-hr was obtained. The specific growth rate was obtained from the least squares slope of a semi-log plot of cell density vs time.

The cultivation data for *N. tabacum* var Wisconsin 38 was comparable to literature data for other varieties of *N. tabacum*. For *N.tabacum* var. Bright Yellow, a lag phase of one day and exponential growth of four to seven days have been reported [Hooker et al., 1989; Hooker et al., 1990]. For *N.tabacum* var. *Xanthi ova* a lag phase of one day and exponential growth phase of nine days was reported [Matsumoto et al., 1970]. Another report indicated a lag phase of two days and an exponential growth phase of six days for *N. tabacum* var. Xanthi [Filner, 1964]. The average maximum cell density for the three runs was 11.51 ± 0.73 g/L on a dry basis. This value is slightly higher than that cited for *N. tabacum* var. *Xanthi ova* of 7.8 g/L.

Growth curves based on PCV and culture conductivity measurements are presented in Figures 5 and 6 respectively. Also presented in Figures 7 and 8 are plots relating dry cell density to PCV and dry cell density to culture conductivity. The linear correlation between culture conductivity and dry cell density cell growth measurements in Figure 8 indicates the potential of conductivity measurements for *in situ* measurement of cell density [Dewey et al. 1989]. The addition of a

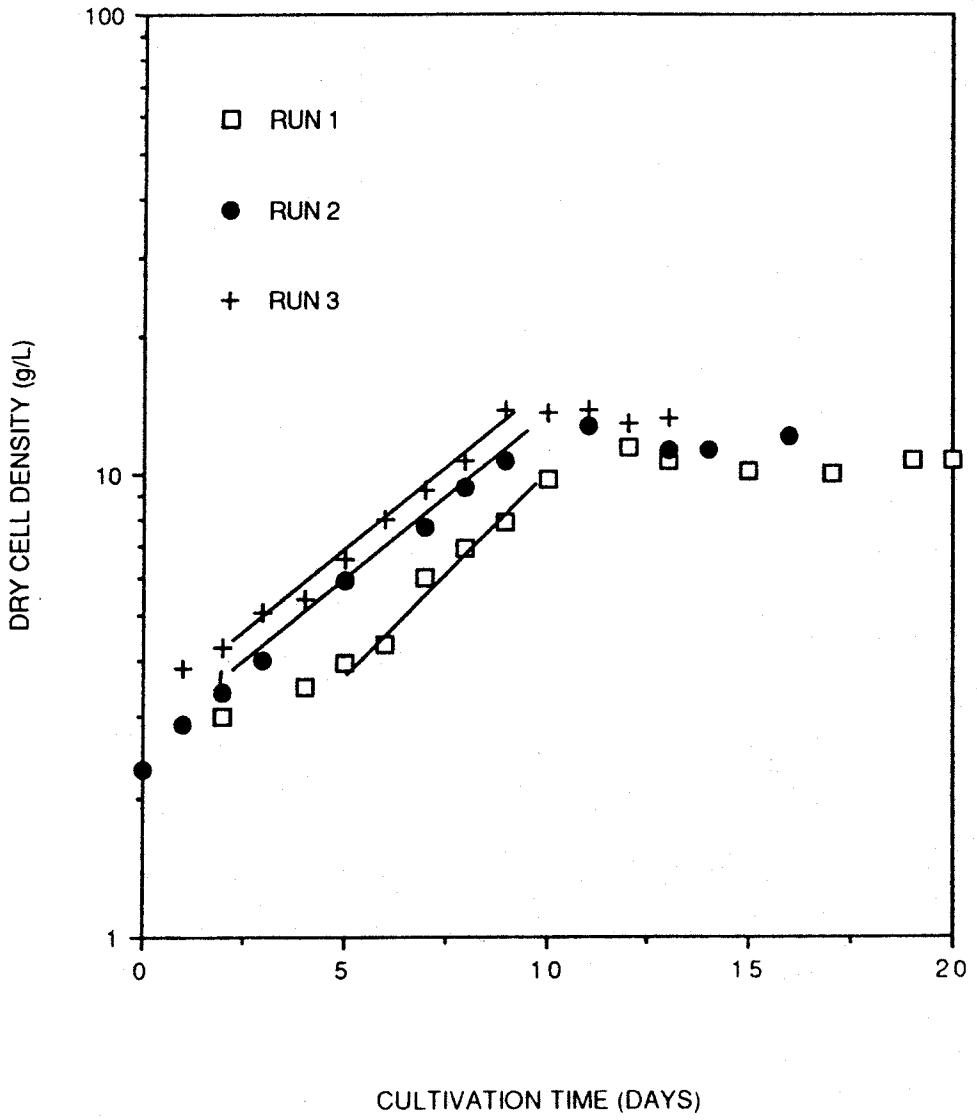

Figure 4. Logarithmic growth curves for shake flask cultures based on dry cell density. The growth rates can be found in Table 8. The average slope equals 0.0689 g/L-hr. Conditions: 27 °C, 150 rpm, 40 mL culture/125 mL flask.

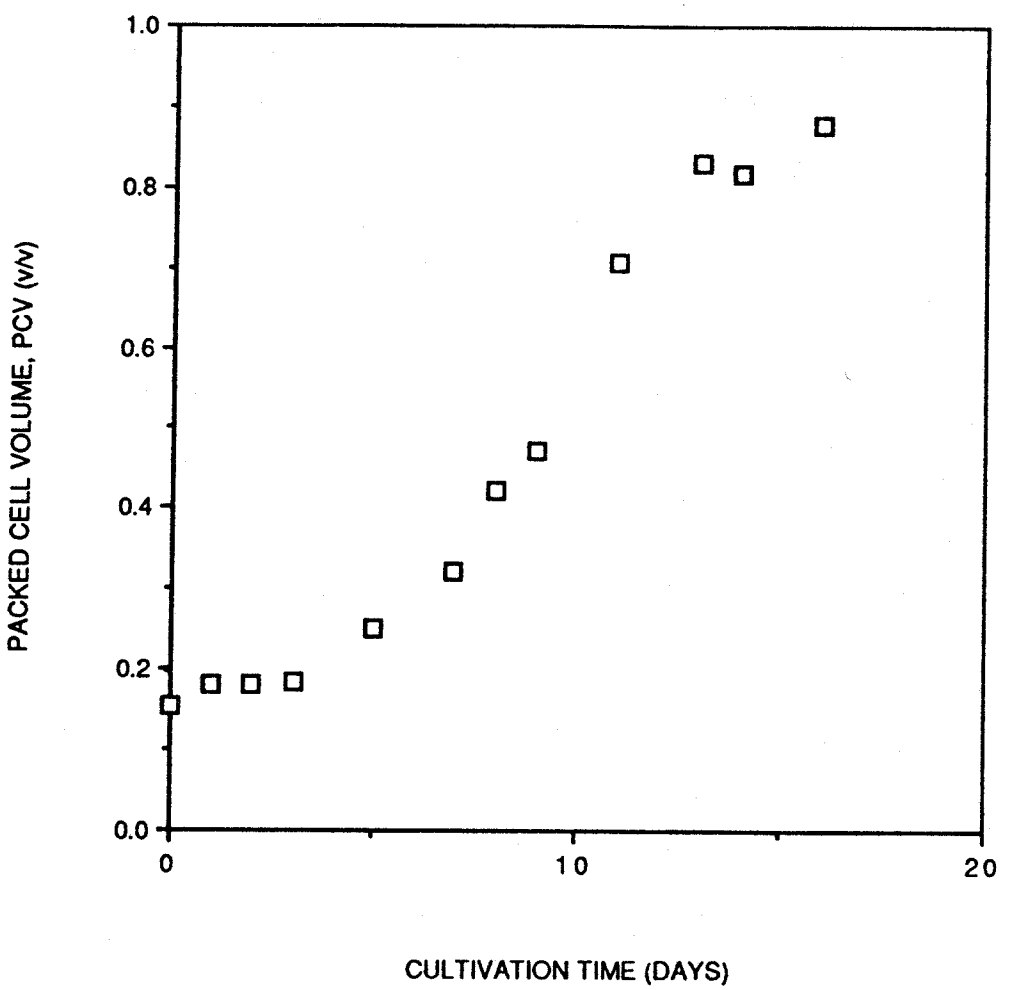

Figure 5. Shake flask growth curve based on packed cell volume (PCV). Conditions: 27 °C, 150 rpm, and 40 mL/125 mL flask.

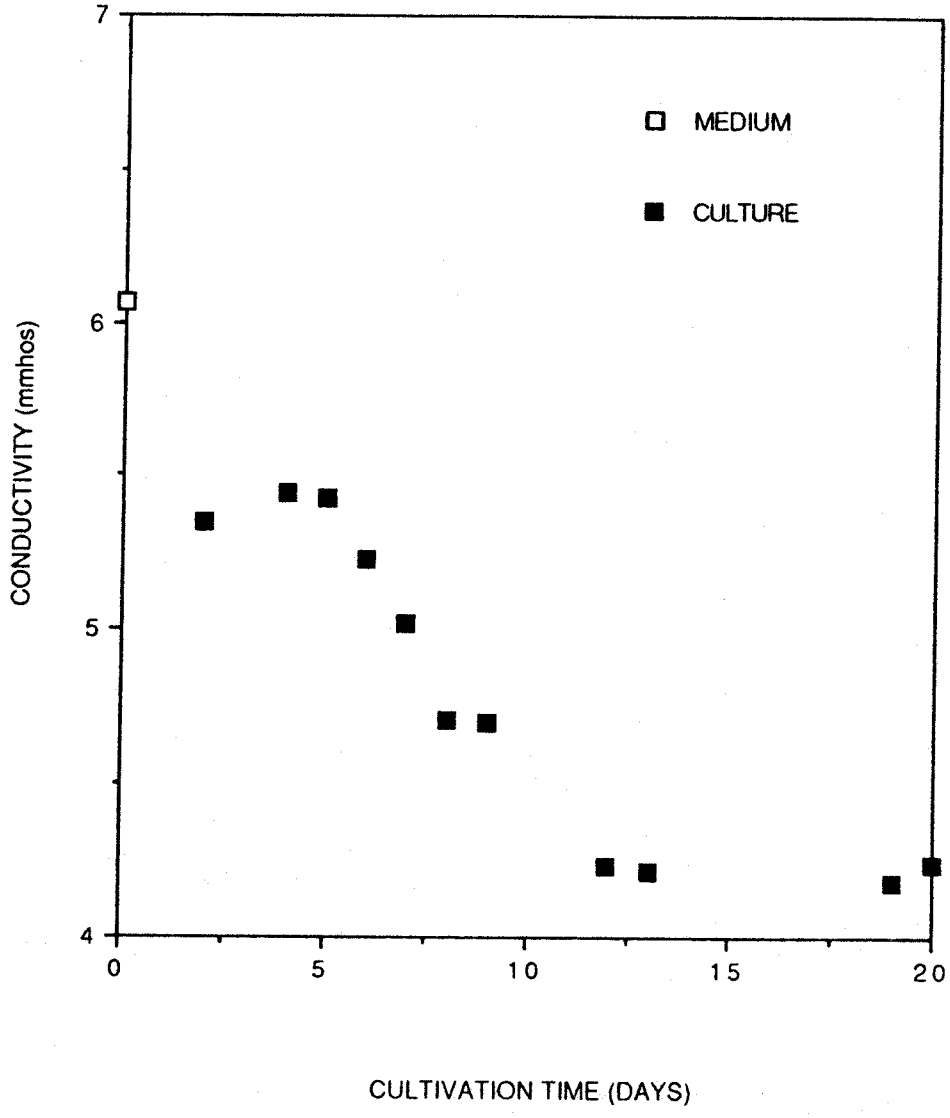

Figure 6. Shake flask growth curve based on conductivity. Conditions: 27 °C, 150 rpm, and 40 mL/125 mL flask.

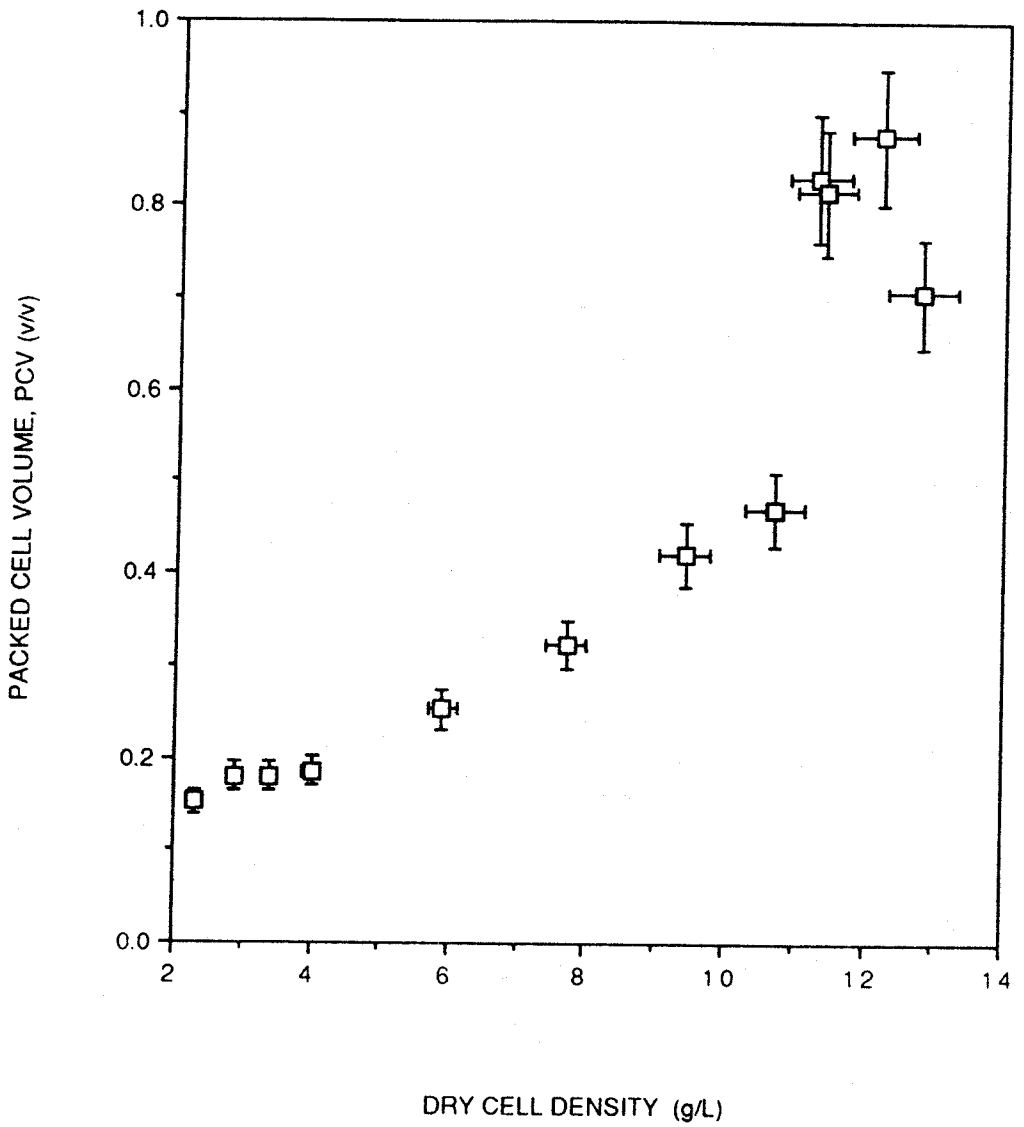

Figure 7. Correlation between the dry cell density and PCV for the shake flask growth curves. Error bars indicate ± 1s for four repeated measurements. Conditions: 27 °C, 150 rpm, 40 mL culture/ 125 mL flask.

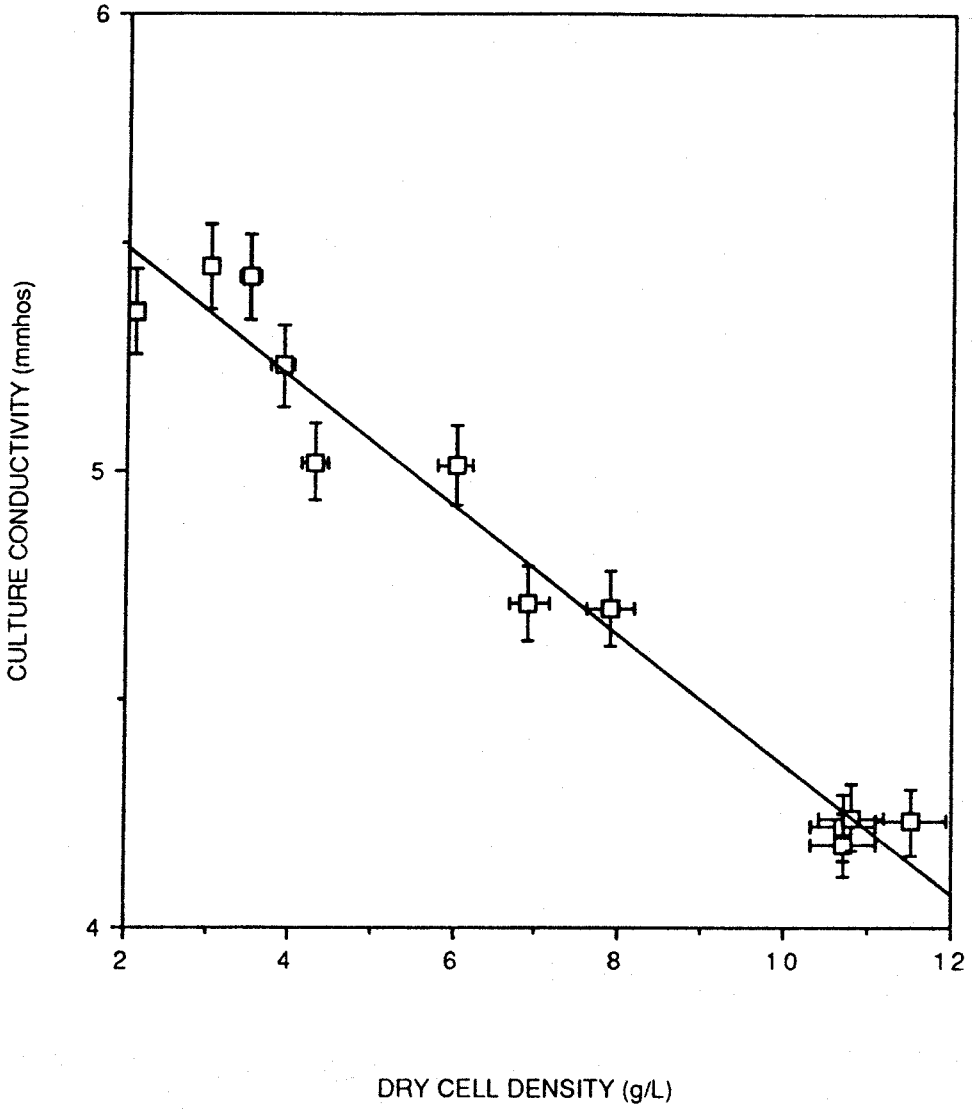

Figure 8. Correlation between the dry cell density and conductivity for the shake flask growth curves. Error bars indicate ± 1s for four repeated measurements. Conditions: 27 °C, 150 rpm, 40 mL culture/ 125 mL flask.

conductivity electrode to the bioreactor for *in situ* culture conductivity measurements would eliminate the need for withdrawing samples to measure biomass growth, thus decreasing the chances of bacterial contamination.

Effect of Sucrose Concentration on Growth Rate and Maximum Cell Density

The effect of initial sucrose concentration on biomass growth rate and density was investigated. Figure 9 shows three growth curves at initial sucrose concentrations of 20, 30, and 40 g/L. Maximum cell densities (X_{max}) and specific growth rates (μ) for each different initial sucrose concentration can be found in Table 8. For comparison, literature values for initial sucrose concentrations (S_o) of 22, 29 and 39 g/L, yielded X_{max} values of 5, 7.8, and 10.4 g/L respectively for *N. tabacum* var. *Xanti ova.* [Matsumoto et al, 1970]. The yield coefficient (Y_o), determined from the slope of X_{max} versus S_o is 0.420 g dry cell/g of initial sucrose (Figure 10). The initial sucrose concentration affects the specific growth rate (μ) by the following equation

$$\mu = \frac{\mu_{max} S_o}{K_S + S_o} \qquad (15)$$

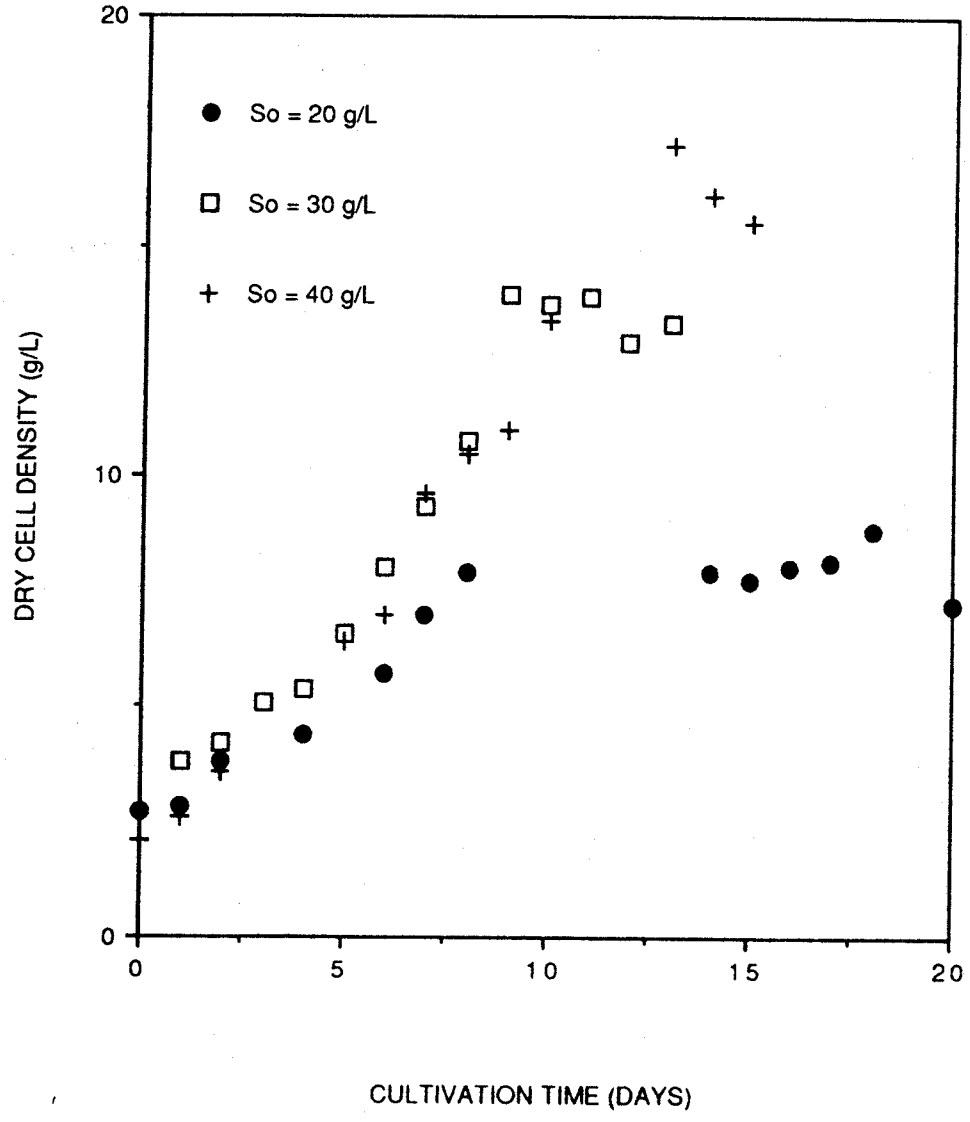

Figure 9. Shake flask culture growth curves for different initial sucrose concentrations. Conditions: 27 °C, 150 rpm, 40 mL culture/ 125 mL flask.

Table 8. Maximum cell density and specific growth rate for different initial sucrose concentrations in shake flask studies. Error bars indicate ± 1s for linear fit in specific growth rate calculations. Conditions: 27 °C, 150 rpm, 125 mL flask.

Initial Sucrose (g/L)	Maximum Cell Density Dry Basis (g/L)	Specific Growth Rate (g/L-hr) ± 1s
20	7.81	0.055 ± 0.004
30	10.67 (run 1)	0.0703 ± 0.005
	11.93 (run 2)	0.0694 ± 0.004
	13.90 (run 3)	0.0697 ± 0.004
40	16.2	0.073 ± 0.005

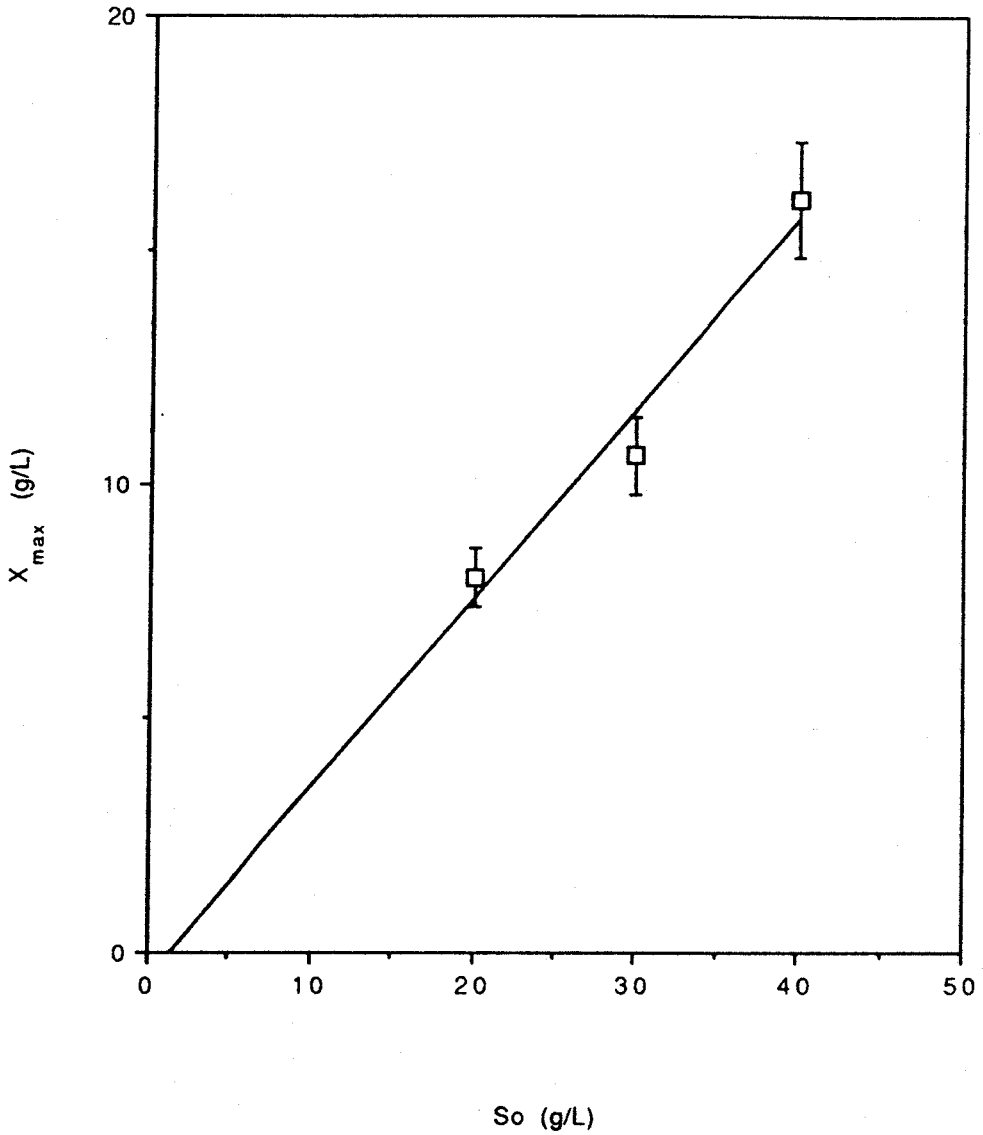

Figure 10. Yield coefficient (Y_o) determination for shake flask study. Y_o equals 0.420 g dry cell/ g of initial sucrose. Error bars indicate ±1s for 3 to 5 averaged X_{max} values. Conditions: 27 °C, 150 rpm, 40 mL culture/ 125 mL flask.

were K_s is the Michaelis constant, and μ_{max} is the maximum specific growth rate. A Lineweaver-Burke plot of $1/\mu$ vs $1/S_o$ is presented in Figure 11. The kinetic parameters obtained from the plot are 22.57 g/L for K_s and 0.1175 g/L-hr for μ_{max}.

Sugar Utilization

The sucrose in the medium is hydrolyzed intercellularly by invertase which is generally confined to the cell wall [Straus, 1961]. By the hydrolytic action of the enzyme, sucrose is hydrolyzed into glucose and fructose. The hydrolysis products are then metabolized by the cell [Brock, 1988]. Sucrose, glucose, and fructose utilization curves during shake flask culturing are presented Figure 12. The total sugar utilization and cell density growth versus culture time is presented in Figure 13. At the end of the cultivation period, the total sugar level was 9.9 g/L.

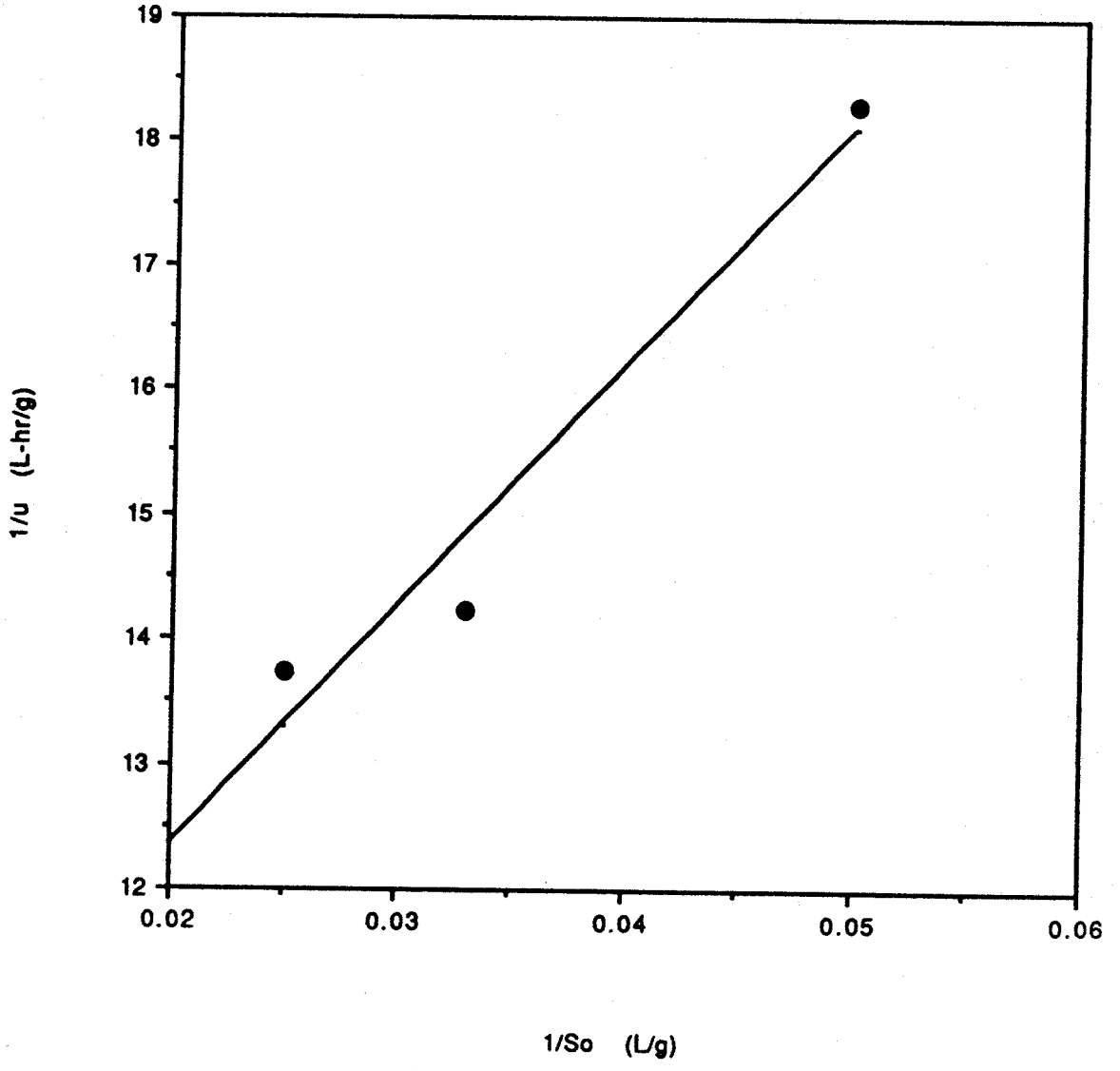

Figure 11. Lineweaver-Burke plot for shake flask culture growth of *Nicotiana tabacum*. μ_{max} = 0.1175 g/L-hr, K_s = 22.56 g/L. Conditions: 27 °C, 150 rpm, 40 mL culture/ 125 mL flask

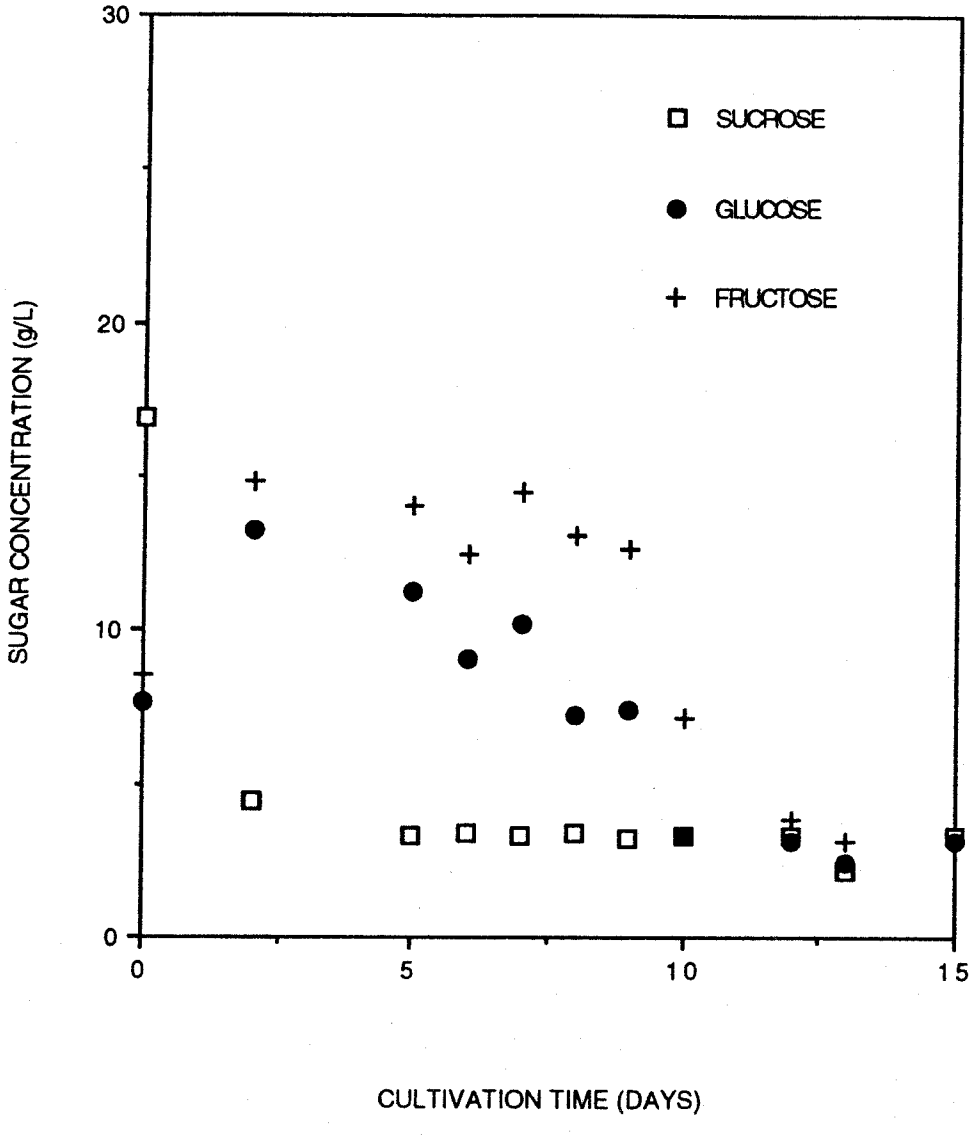

Figure 12. Shake flask sugar utilization curves. Conditions: 27 °C, 150 rpm, and 40 mL/125 mL flask.

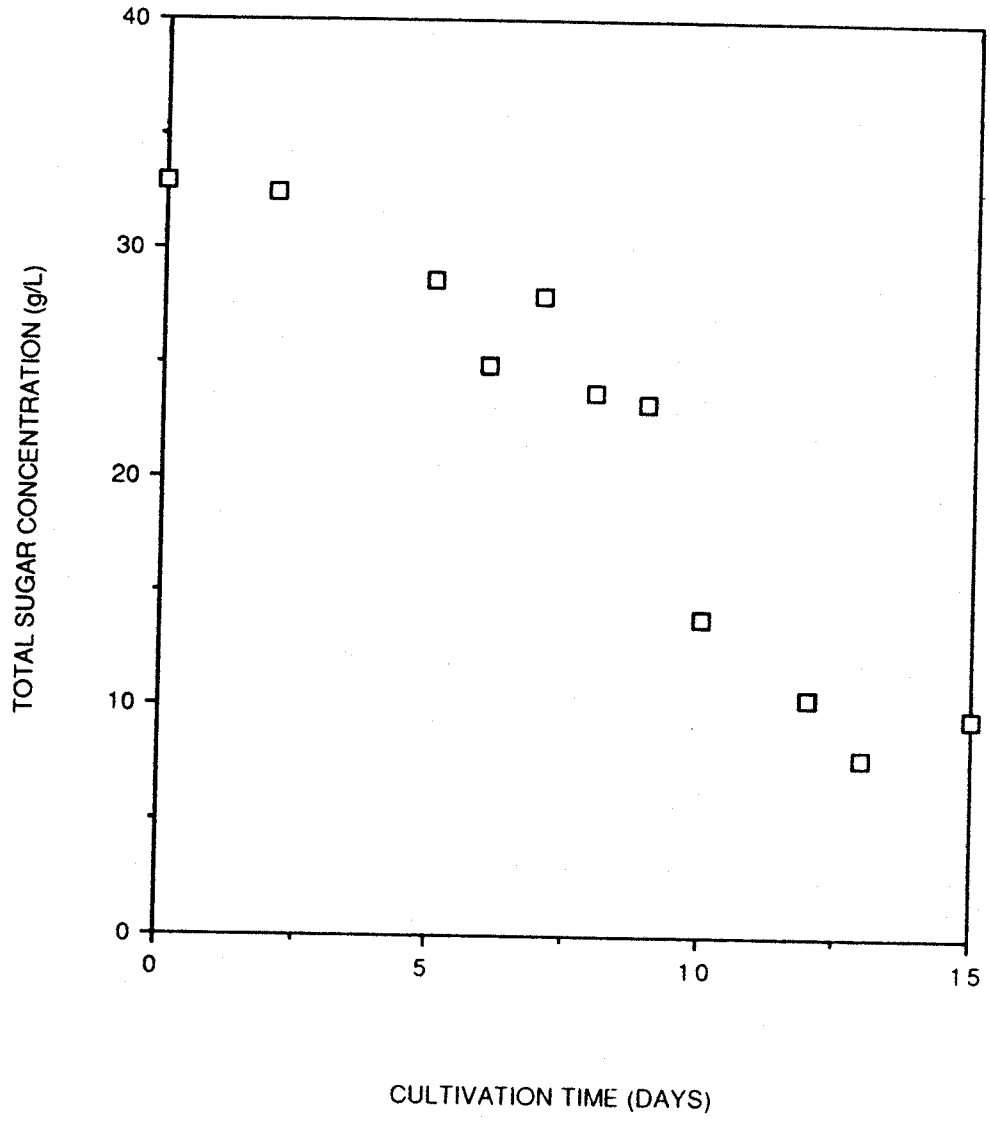

Figure 13. Shake flask total sugar utilization curve. Conditions: 27 oC, 150 rpm, and 40 mL/125 mL flask.

BIOREACTOR STUDIES

An *in situ* method for estimate of k_La in an aerobic stirred-tank bioreactor was investigated. The range of process variables which provided suitable initial k_La values in liquid medium were first determined. Mass transfer model assumptions were then validated through additional experiments. Actual cultivation of tobacco cells in a two-liter stirred-tank bioreactor was repeated three times. *In situ* mass transfer estimates focused on estimation of Q_o and k_La as a function of culture time.

Process Variables

An aerated stirred-tank bioreactor was chosen for study because previous work indicates that high cell densities can be obtained [Kato et al., 1971; Hooker et al.,1990]. Production of plant cells in a stirred-tank bioreactor requires acceptable settings for these process parameters: temperature, impeller design, agitation rate, and air flow rate. The temperature for all bioreactor runs was set at 27 °C, consistent with the shake flask studies. Three different impeller blades reported in the literature were considered: turbine blade [Kato et al.,1975], marine blade [McDonald and Jackson,1989], and paddle blade [Hooker et al.,1990]. Each different impeller system used in the bioreactor is listed in Table 9 alongwith the corresponding process parameters. For bioreactor runs involving either a turbine blade impeller or a marine blade impeller, considerable

Table 9. Initial bioreactor runs.

Run #	Impeller System	Agitation Rate (rpm)	Air Flow Rate (LPM)	Initial k_La (hr^{-1})
1	Turbine	70	1.5	5.621
2	Marine Blade	70	0.7	
3	Marine Blade	150	0.7	1.100

cell damage resulted and no biomass growth was observed. In previous work, both turbine blade impellers and marine blade impellers have proven effective in stirred-tank bioreactors for high density cell cultivation [Kato et al., 1975; Hooker et al., 1990; McDonald and Jackson,1989]. Previously reported findings and this study used the same process variables, thus indicating that the variation of tobacco cells used in this study are more susceptible to hydrodynamic stress. Due to failure with both the turbine impeller and the marine blade impeller, a paddle impeller was implemented as recommended by Hooker et al. [1990] and Kato et al. [1975] for maximum biomass production. The agitation rate for the impeller, 150 rpm, was chosen based on previous cultivation studies [Hooker et al., 1990]. The air flow was set at 0.7 L/min (0.44 v/v) in order to maintain a dissolved oxygen concentration of at least 0.075 mmole O_2/L (30% of saturation) in the cell culture. Table 6 list the bioreactor process conditions when using the paddle impeller.

The effects of impeller design, agitation rate, and air flow rate on oxygen mass transfer can be correlated as a single variable, the mass transfer coefficient (k_La). The initial k_La values for the bioreactor runs when only medium was present using the turbine and marine blade impellers discussed above can be found also in Table 9. The initial k_La value ranges from 1.1 to 5.6 hr^{-1} for the unsuccessful runs. The initial k_La for the chosen parameters (paddle impeller, 150 rpm, and 0.7 L/min. air flow) is 7.72 ± 0.13 (1s) hr^{-1}. Kato et al. [1975] reports initial

k_La values greater than 10 hr^{-1} are required for maximum cell density. For the three impellers investigated, the paddle impeller produced the highest initial k_La value for the bioreactor. Both Hooker et al. [1990] and Kato et al. [1975] findings indicate that the paddle impeller provides the best opportunity for obtaining high density cell cultivation in the 2 L stirred-tank bioreactor.

Analysis of Model Assumptions

During the step change in air flow necessary for estimation of k_La, the dissolved oxygen concentration changes rapidly. A response lag time for the dissolved oxygen electrode can result, and the electrode output is no longer directly related to the instantaneous value of the oxygen concentration in the bioreactor. This lag time is mainly due to diffusion of oxygen through the membrane [Ruchti et al., 1981], and can be represented by a first order approximation, equation (14). If the electrode response is fast, i.e. $\tau_E < 1/k_La$, then the lag time is negligible [Van't Riet, 1979]. For τ_E values less than or equal to $1/k_La$, an error of less than 6% is reported. At the process parameters discussed above, a time constant of 21.6 $\pm$ 1.0 seconds was determined for the VirTis galvanic oxygen electrode. This value is two orders of magnitude larger than the initial $1/k_La$ in the reactor, thus validating the assumption of negligible electrode response delay.

At low respiration rates, the dissolved oxygen concentration vs time data curved upward slightly. Deviations in the expected linearity of this curve were due to surface mixing. Nitrogen was sparged over the medium in the reactor to minimize surface aeration. An initial drop in the D.O. reading of about 30 % of full scale resulted. Figure 14 shows the curve representing the change in steady state values. Figure 15 shows the time dependent change in oxygen for a step change in the reactor aeration while nitrogen is sparged over the surface, and another curve representing the step change without the nitrogen blanket.

The initial k_La measurements on the medium were affected by the difference between the oxygen concentration in equilibrium with the gas phase and the actual dissolved oxygen concentration (driving force). The oxygen concentration in equilibrium with the gas phase for the medium was found by assuming both Henry's law applies and that the saturated oxygen concentration is the same at all points in the gas-liquid dispersion (ideally mixed gas phase). Both the saturated oxygen concentration and Henry's law value for water and medium at 27 ^oC and 0.21 atm can be found in Table 10. The saturated medium dissolved oxygen concentration was found by calibrating the D.O. electrode to 100% for saturated water at 27 ^oC and measuring the % D.O. in the saturated medium.

The concept of an ideally mixed gas phase in the liquid may not truly depict the actual physical situation. The presence of dissolved salts found in the medium can hinder the coalescence

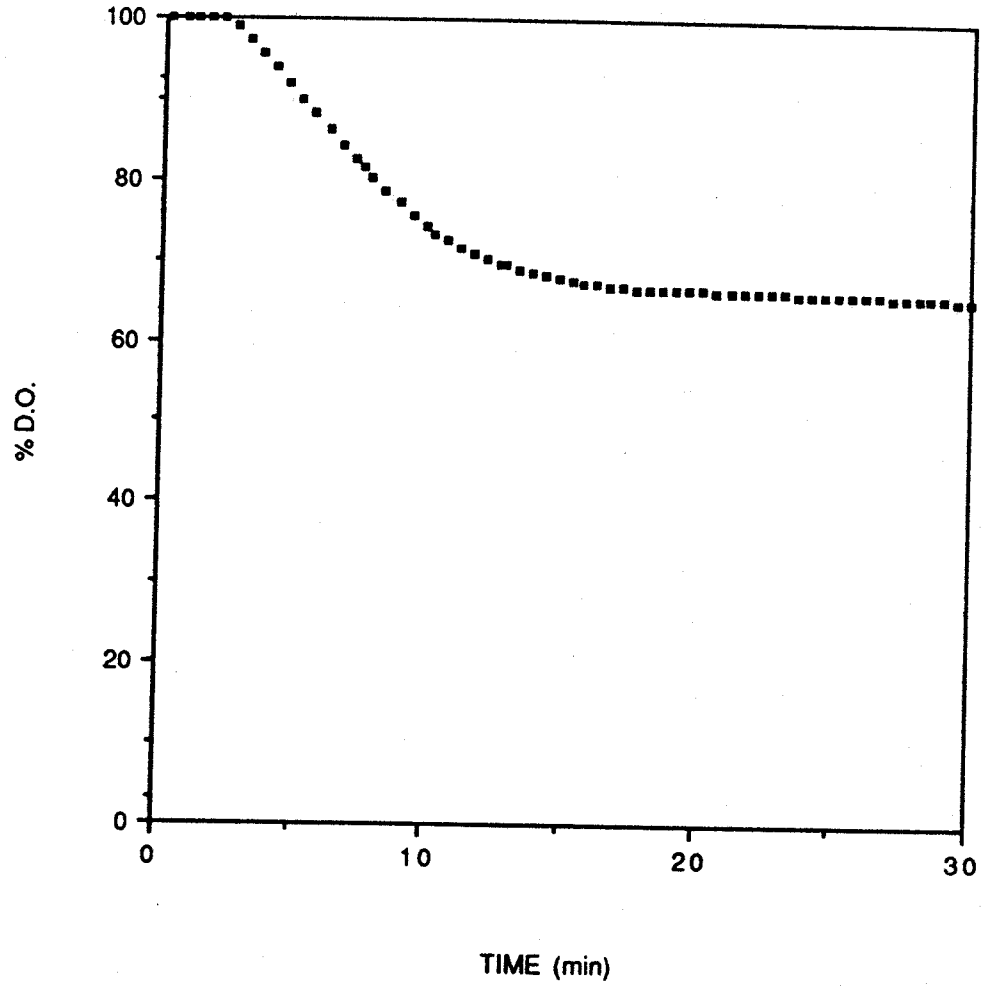

Figure 14. Change in steady state caused by introducing a blanket of nitrogen over the surface of the medium.
Cultivation Conditions:

total liquid volume = 1600 mL	150 rpm
inoculum volume = 300 mL	27 °C
age of inoculum = 8 days	paddle impeller
nitrogen flow = 1.14 L/min.	
air flow = 0.7 L/min.	

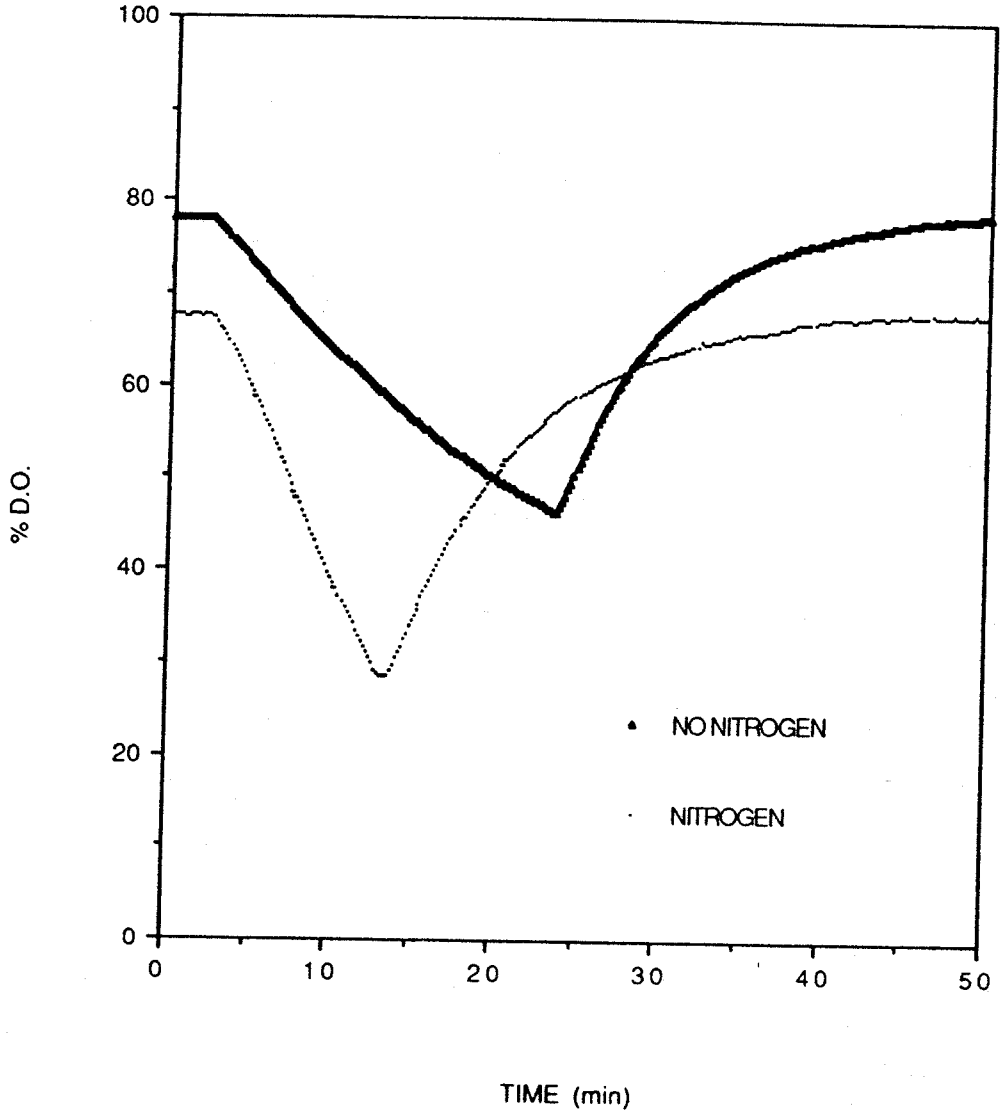

Figure 15. Two curves depicting the time dependent change in oxygen for a step change in the bioreactor aeration. One curve represents the step change when nitrogen was blanketed over the surface, while the other represents the step change without a nitrogen blanket.

Cultivation Conditions:

total liquid volume = 1600 mL	150 rpm
inoculum volume = 300 mL	27 °C
age of inoculum = 8 days	paddle impeller
nitrogen flow = 1.14 L/min.	
air flow = 0.7 L/min.	

Table 10. Henry's law values and saturated dissolved oxygen concentrations for water and medium while in the bioreactor.

Liquid	H (atm/mmole O_2/L)	C_A^* (mmole O_2/L)
Water	0.815	0.2576
Medium	0.822	0.2555

of gas bubbles [Linek et al.,1982]. This changes the residence times for individual bubbles and invalidates the ideally-mixed assumption and can result in a low k_La value. However, Linek et al. [1982] found that for k_La values less than 36 hr^{-1} the assumption of an ideally mixed gas phase is validated. An initial k_La value of 7.72 hr^{-1} was determined for the 2 L stirred-tank reactor at an air flow rate of 0.7 L/min and an agitation rate of 150 rpm, thus justifying the assumption of a well dispersed gas phase in the medium.

Growth Rate Measurements

The cell biomass in the bioreactor was measured as a function of culture time. The three techniques for measuring cell biomass in the shake flask studies were also used for the bioreactor studies: dry cell density, packed cell volume, and conductivity. Figure 16 shows the growth curve based on dry cell density. The culture increased in dry cell density until day seven and then sharply decreased. Similar results were observed for the packed cell volume vs. time curve in Figure 17. Conductivity decreased gradually through out the run as can be seen in Figure 17. The specific growth rate based on semi-log plot of dry cell density and normalized to X_{max} (4.12 g/L) was only 0.0054 hr^{-1}.

The sharp decrease in the bioreactor cell biomass relative to the shake flasks indicates the possibility of shear damage. While the biomass growth rates were comparable for both bioreactor and shake flask studies up until day seven, they differ

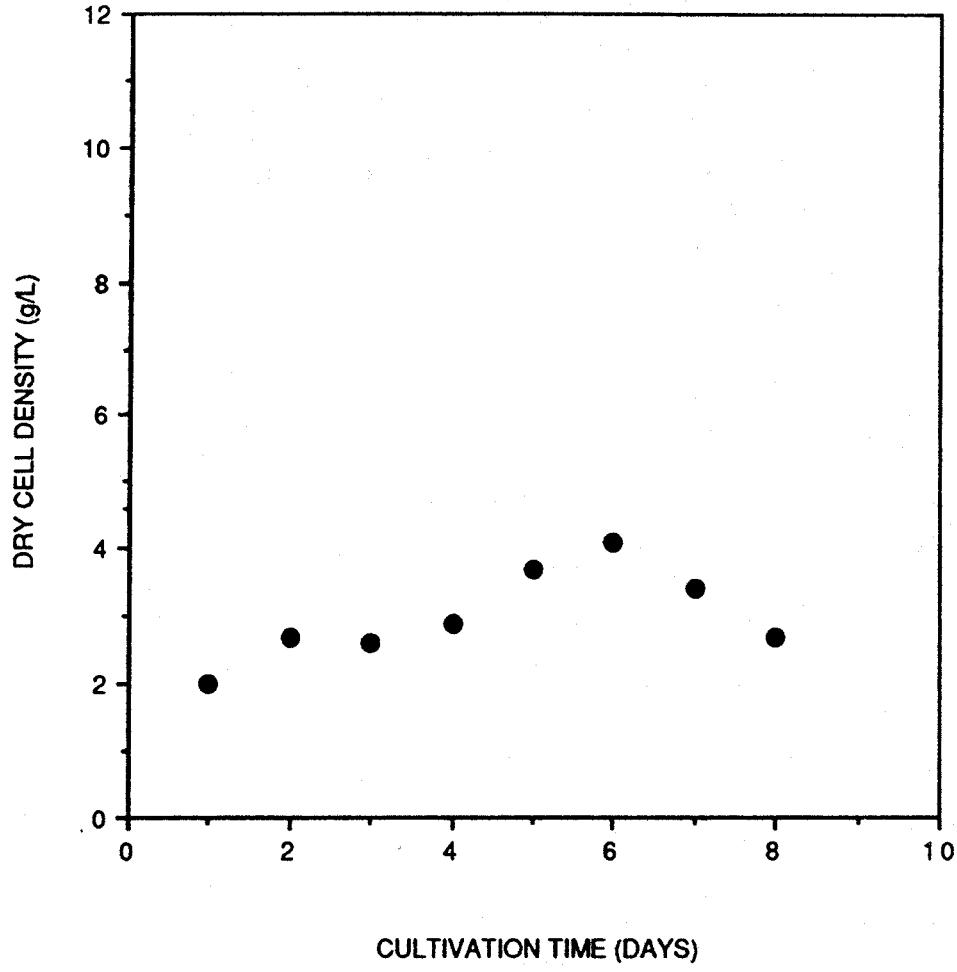

Figure 16a. Bioreactor growth curve based on dry cell density, run 1.
Cultivation Conditions:

total liquid volume = 1600 mL	150 rpm
inoculum volume = 300 mL	27 °C
age of inoculum = 8 days	paddle impeller
nitrogen flow = 1.14 L/min.	
air flow = 0.7 L/min.	

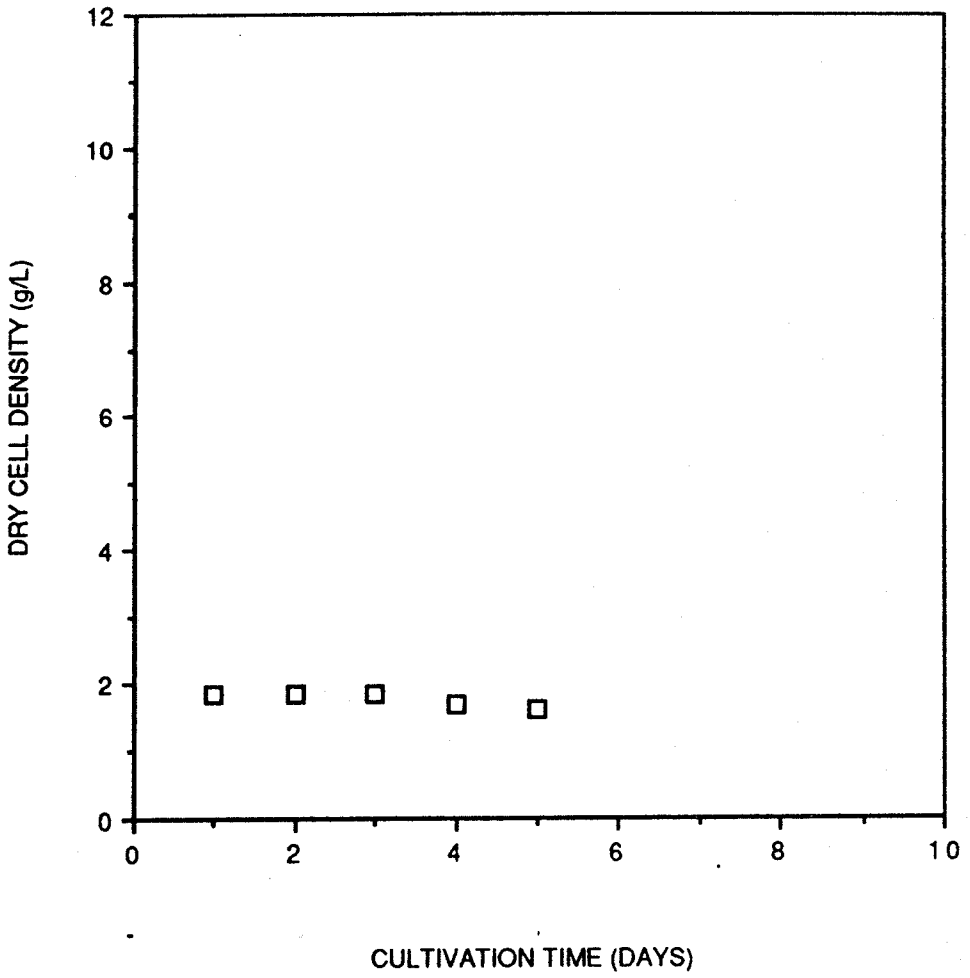

Figure 16b. Bioreactor growth curve based on dry cell density, run 2.
Cultivation Conditions:

total liquid volume = 1600 mL	150 rpm
inoculum volume = 300 mL	27 °C
age of inoculum = 8 days	paddle impeller
nitrogen flow = 1.14 L/min.	
air flow = 0.7 L/min.	

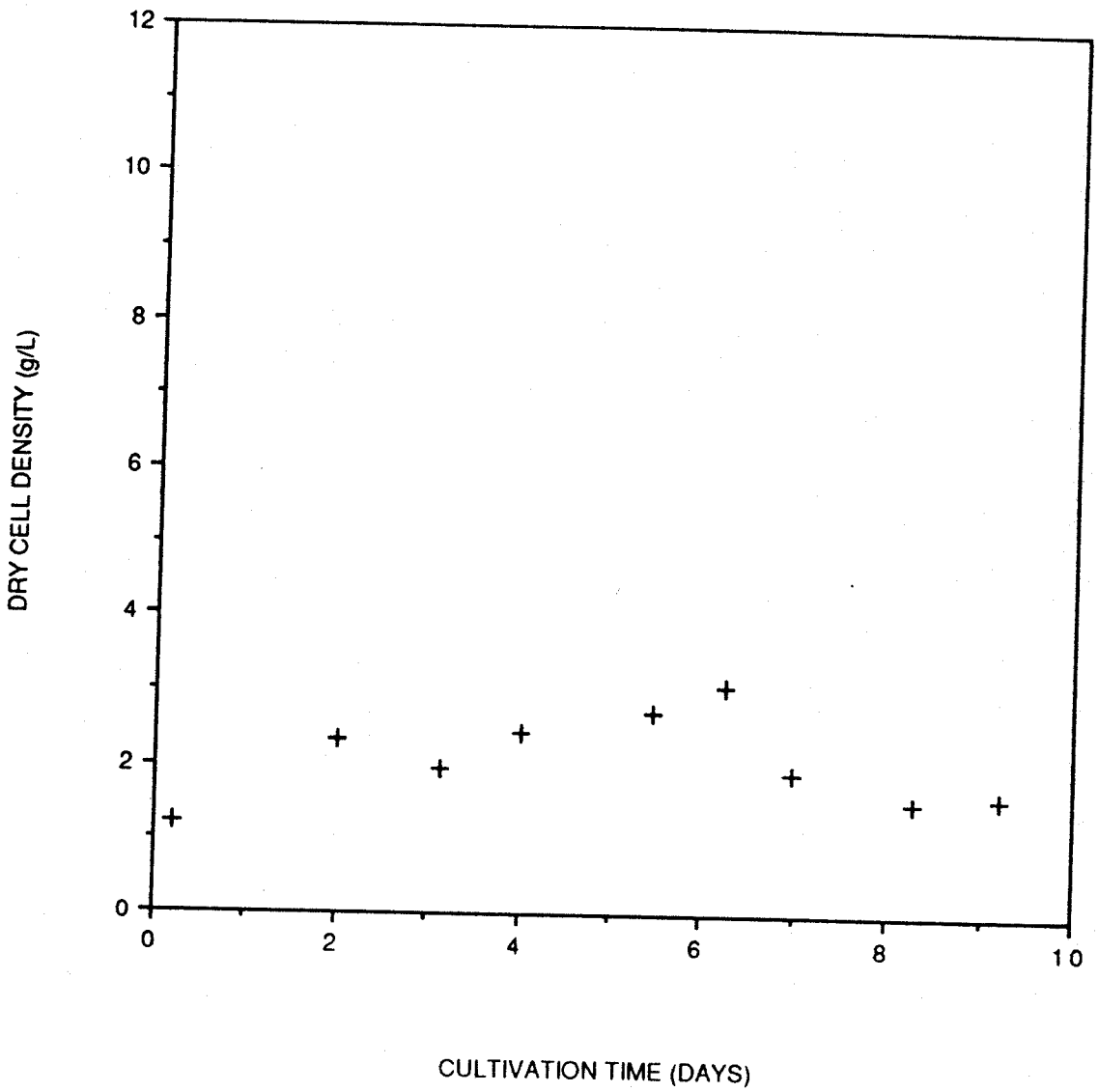

Figure 16c. Bioreactor growth curve based on dry cell density, run 3.
Cultivation Conditions:
total liquid volume = 1800 mL
inoculum volume = 500 mL
age of inoculum = 8 days
air flow = 0.8 L/min.
150 rpm
27 °C
paddle impeller

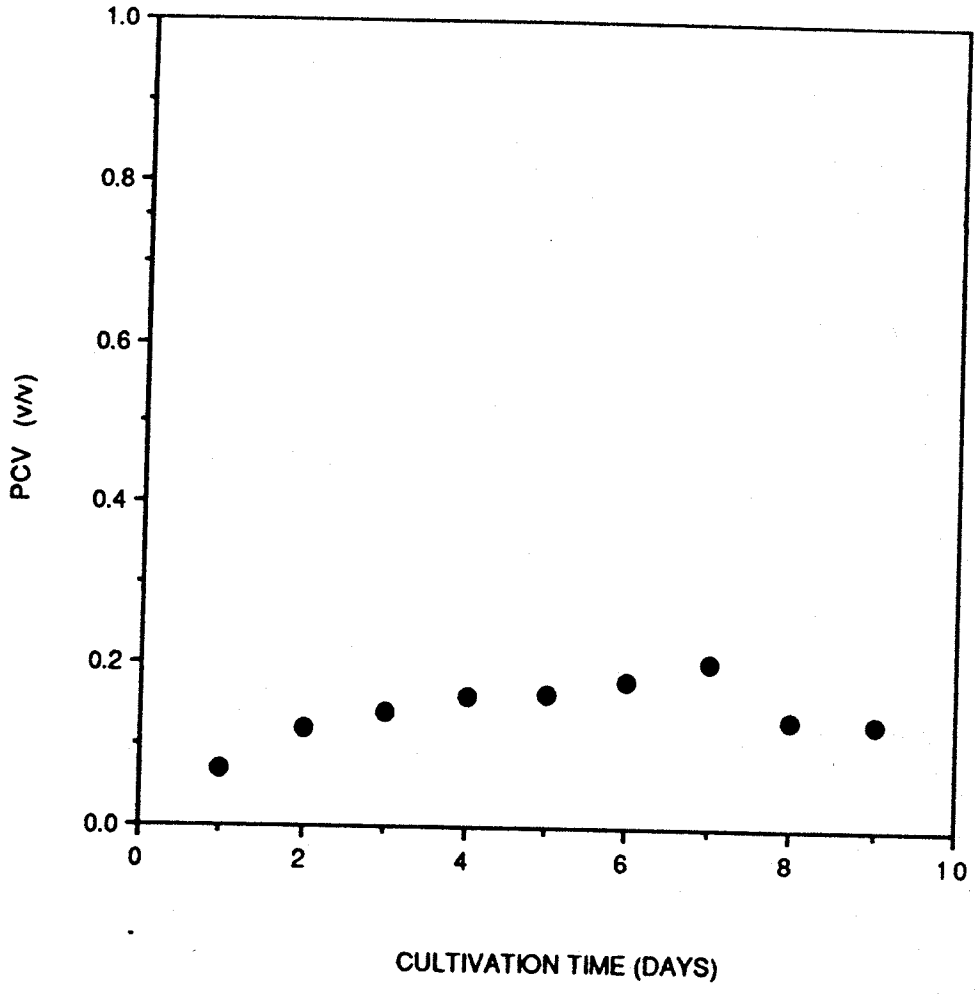

Figure 17a. Bioreactor growth curve based on PCV, run 1.
Cultivation Conditions:

total liquid volume = 1600 mL	150 rpm
inoculum volume = 300 mL	27 °C
age of inoculum = 8 days	paddle impeller
nitrogen flow = 1.14 L/min.	
air flow = 0.7 L/min.	

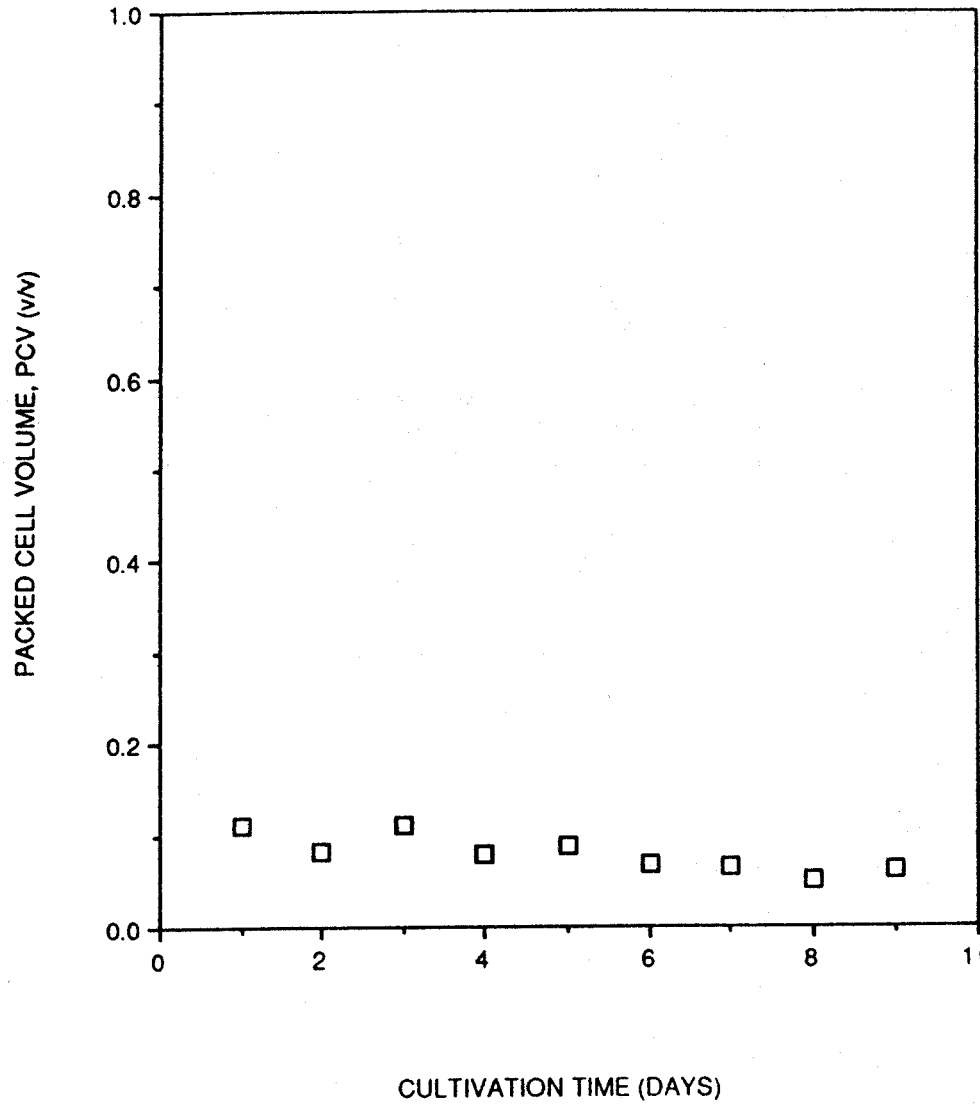

Figure 17b. Bioreactor growth curve based on PCV, run 2.
Cultivation Conditions:

total liquid volume = 1600 mL	150 rpm
inoculum volume = 300 mL	27 °C
age of inoculum = 8 days	paddle impeller
nitrogen flow = 1.14 L/min.	
air flow = 0.7 L/min.	

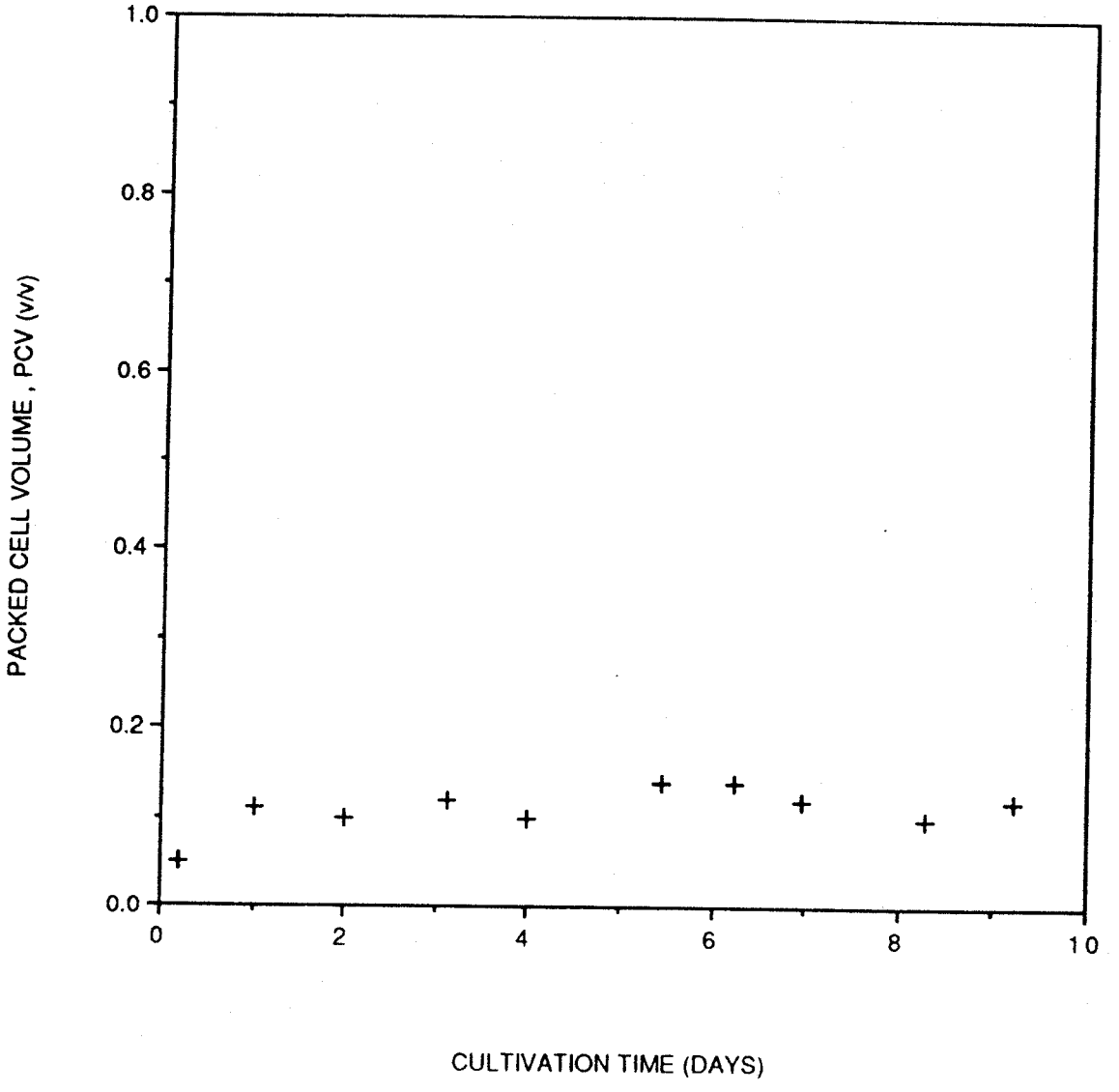

Figure 17c. Bioreactor growth curve based on PCV, run 3.
Cultivation Conditions:

total liquid volume = 1800 mL	150 rpm
inoculum volume = 500 mL	27 °C
age of inoculum = 8 days	paddle impeller
air flow = 0.8 L/min.	

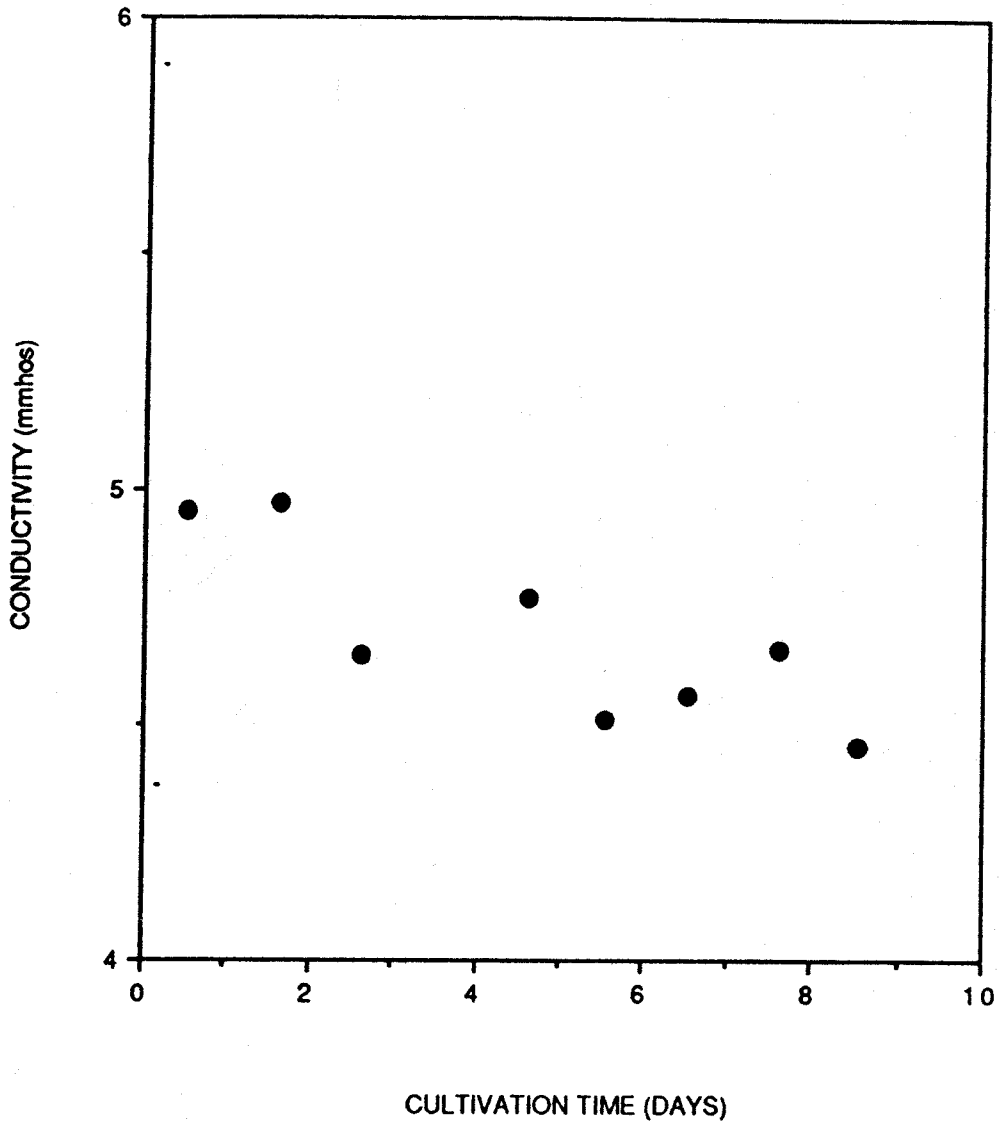

Figure 17d. Bioreactor growth curve based on conductivity, run 1.
Cultivation Conditions:

total liquid volume = 1600 mL	150 rpm
inoculum volume = 300 mL	27 °C
age of inoculum = 8 days	paddle impeller
nitrogen flow = 1.14 L/min.	
air flow = 0.7 L/min.	

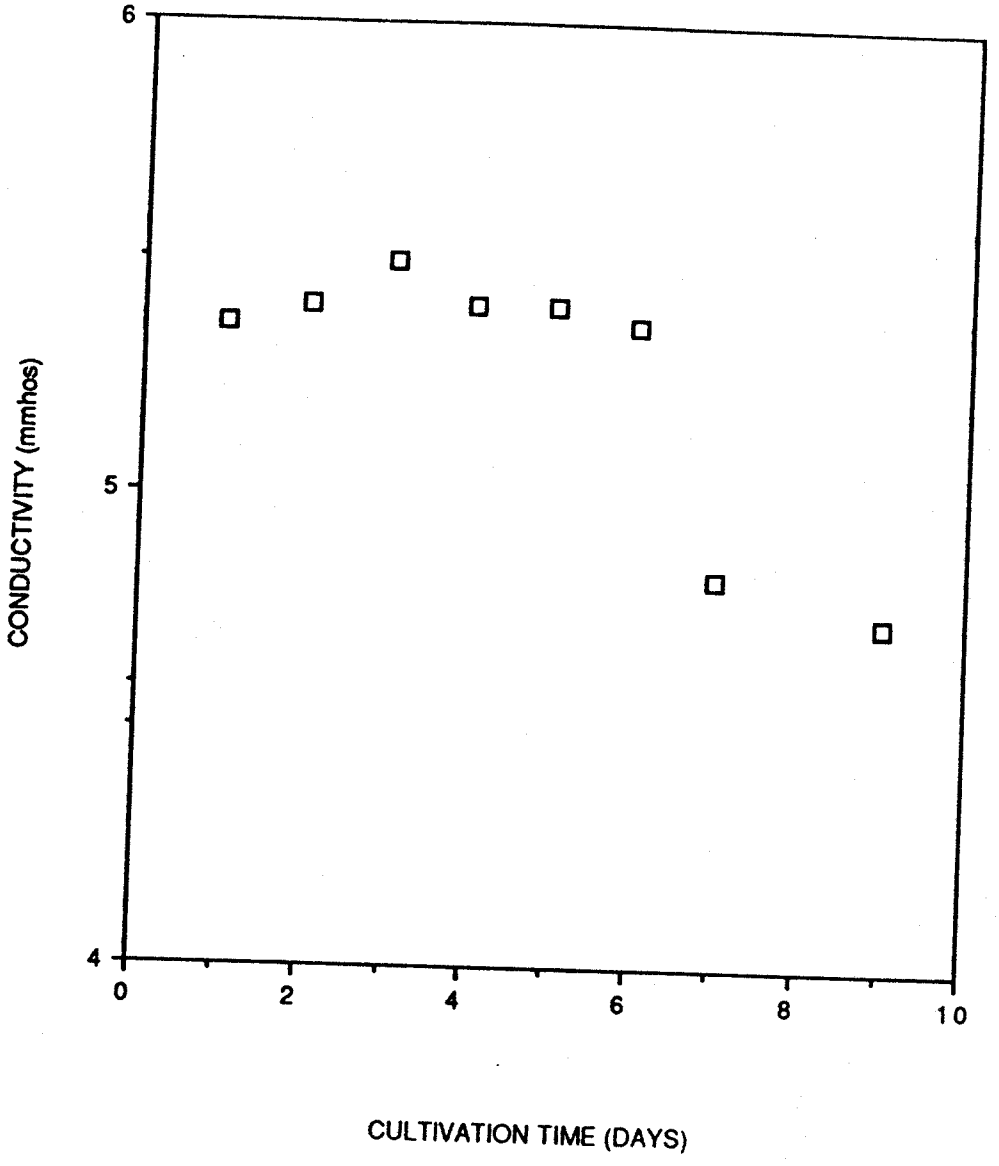

Figure 17e. Bioreactor growth curve based on conductivity, run 2.
Cultivation Conditions:
total liquid volume = 1600 mL
inoculum volume = 300 mL
age of inoculum = 8 days
nitrogen flow = 1.14 L/min.
air flow = 0.7 L/min.
150 rpm
27 °C
paddle impeller

significantly afterwards (Figure 18). The cellular growth rate for the shake flask studies during days six and seven occurred during the later stage of the exponential growth phase. According to shear sensitivity studies by Hooker et al. (1989), cultures in the later stages of exponential growth are more susceptible to shear damage. The notable decrease in cell biomass indicates that exposure to shear generated by the paddle-type impeller possibly ruptured some of the plant cells. The decrease occurred during the exponential growth phase which further validates the possibility of high shear in the bioreactor during the cultivation process.

Microscopic analysis of the culture broth showed an increase in cell deformation, cell rupture, and cell debris. Figure 19 shows the number of cells, fragments, and aggregates as a function of cultivation time. As the cultivation proceeded, the number of fragments increased while the number of single cells decreased. Furthermore, cell aggregates became larger over time and constituted the major fraction of viable biomass after day six. Both the cell biomass growth measurements and the microscopic study provide evidence of shear damage which limited biomass cultivation growth in the bioreactor.

Estimates of Q_o and k_La

Two variations on the *in situ* method for estimates for Q_o and k_La were used. In the first method, a nitrogen blanket was sparged over the surface of the culture. Estimates for Q_o and k_La

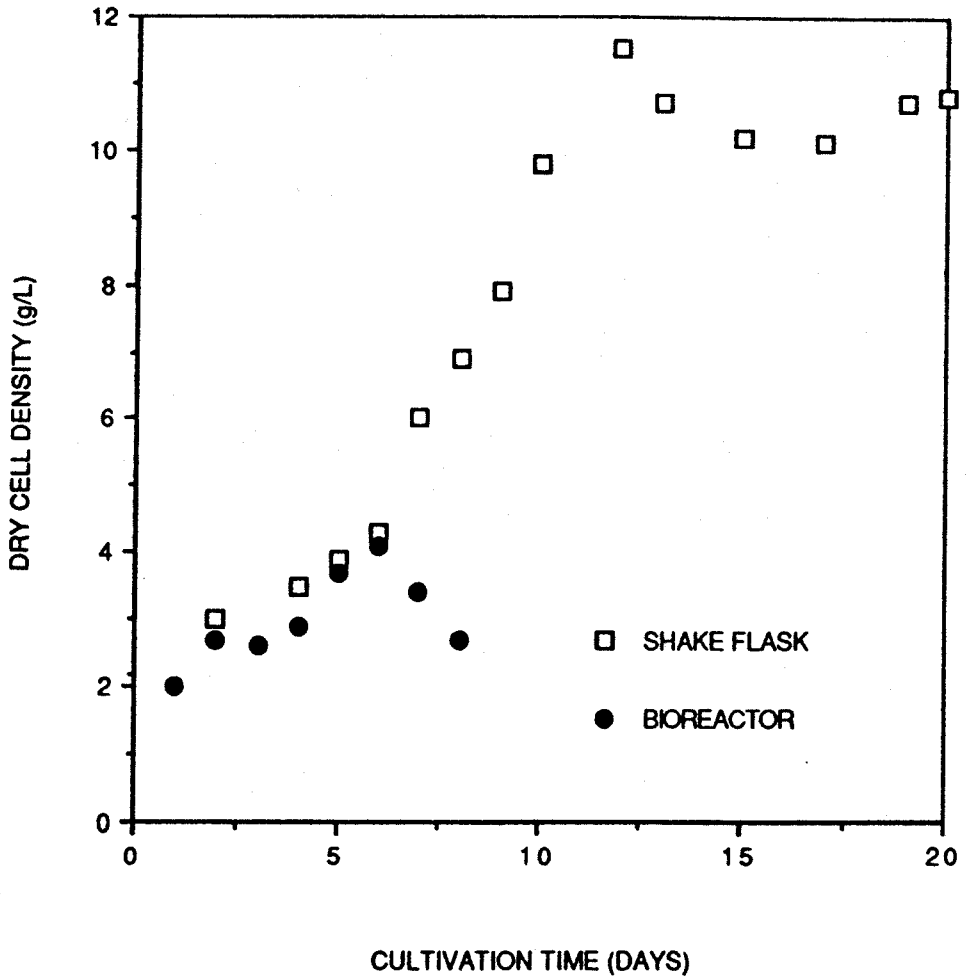

Figure 18a. Bioreactor and shake flask growth curves based on dry cell density, run 1.
Shake Flask Cultivation Conditions:
27°C 150 rpm
flask size = 125 mL total liquid volume = 40 mL
inoculum volume = 10 mL age of inoculum = 8 days
Bioreactor Cultivation Conditions:
total liquid volume = 1600 mL 150 rpm
inoculum volume = 300 mL 27 °C
age of inoculum = 8 days paddle impeller
nitrogen flow = 1.14 L/min.
air flow = 0.7 L/min

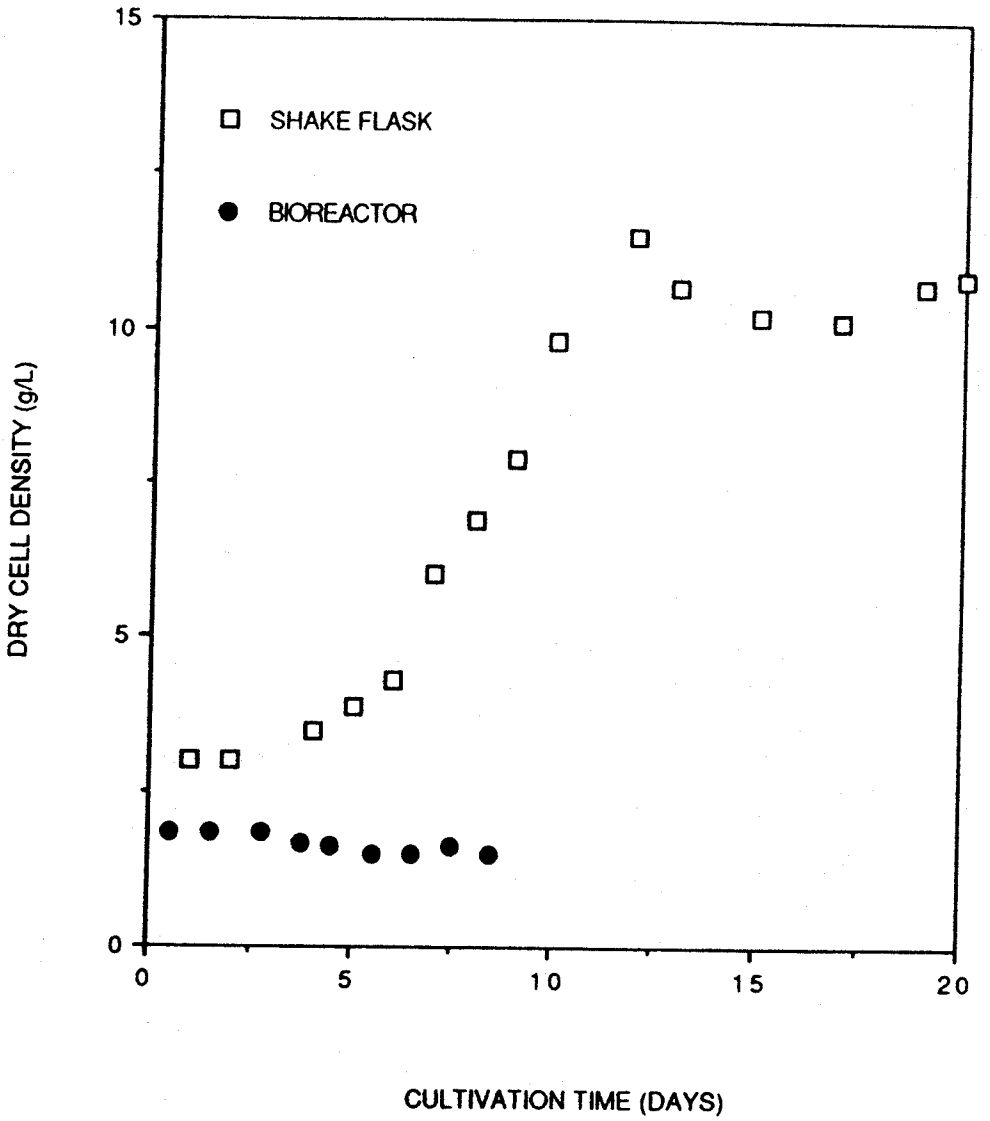

Figure 18b. Bioreactor and shake flask growth curves based on dry cell density, run 2.
Shake Flask Cultivation Conditions:
27°C 150 rpm
flask size = 125 mL total liquid volume = 40 mL
inoculum volume = 10 mL age of inoculum = 8 days
Bioreactor Cultivation Conditions:
total liquid volume = 1600 mL 150 rpm
inoculum volume = 300 mL 27 °C
age of inoculum = 8 days paddle impeller
nitrogen flow = 1.14 L/min.
air flow = 0.7 L/min.

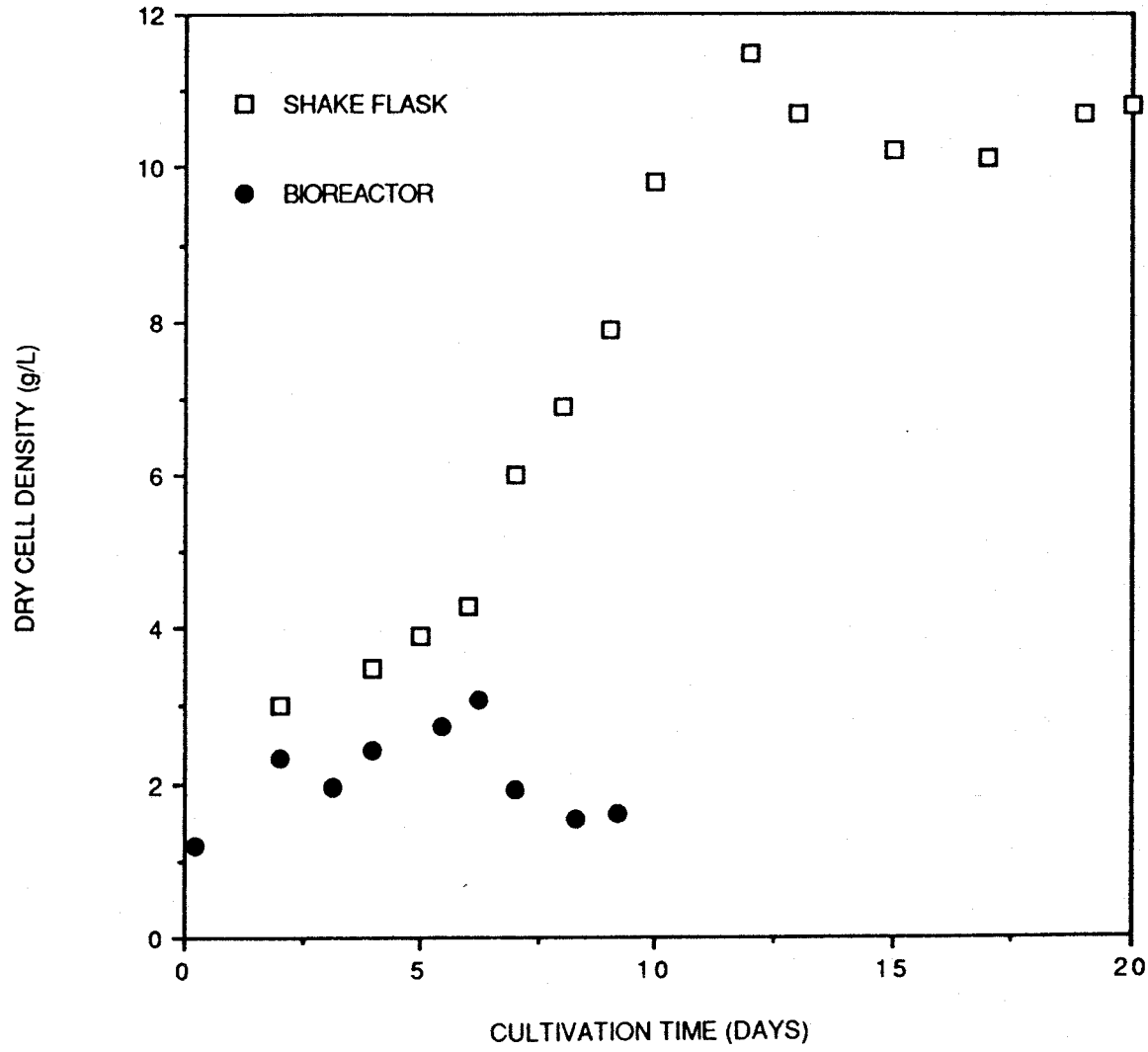

Figure 18c. Bioreactor and shake flask growth curves based on dry cell density, run 3.
Shake Flask Cultivation Conditions:
27°C 150 rpm
flask size = 125 mL total liquid volume = 40 mL
inoculum volume = 10 mL age of inoculum = 8 days
Bioreactor Cultivation Conditions:
total liquid volume = 1800 mL 150 rpm
inoculum volume = 500 mL 27 °C
age of inoculum = 8 days paddle impeller
air flow = 0.8 L/min.

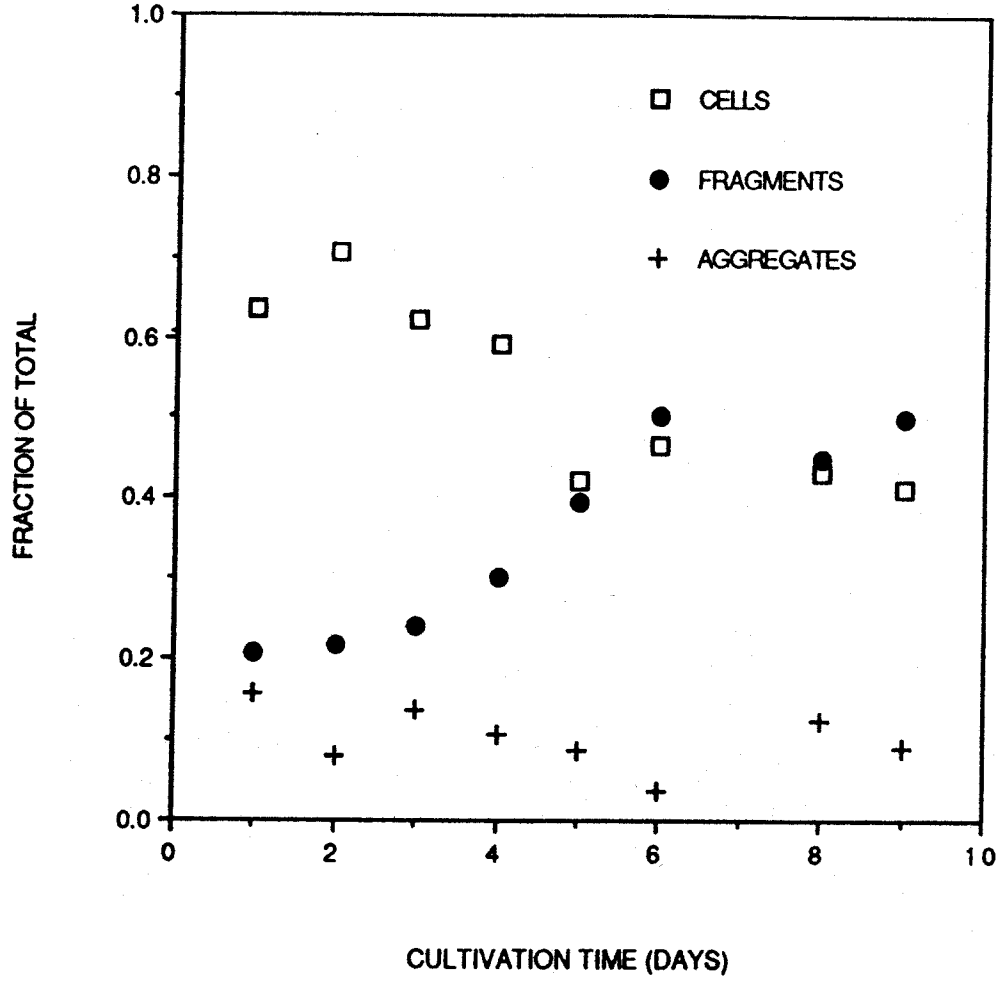

Figure 19a. Bioreactor particle counts based on microscopic analysis, run 1
Cultivation Conditions:

total liquid volume = 1600 mL	150 rpm
inoculum volume = 300 mL	27 °C
age of inoculum = 8 days	paddle impeller
nitrogen flow = 1.14 L/min.	
air flow = 0.7 L/min.	

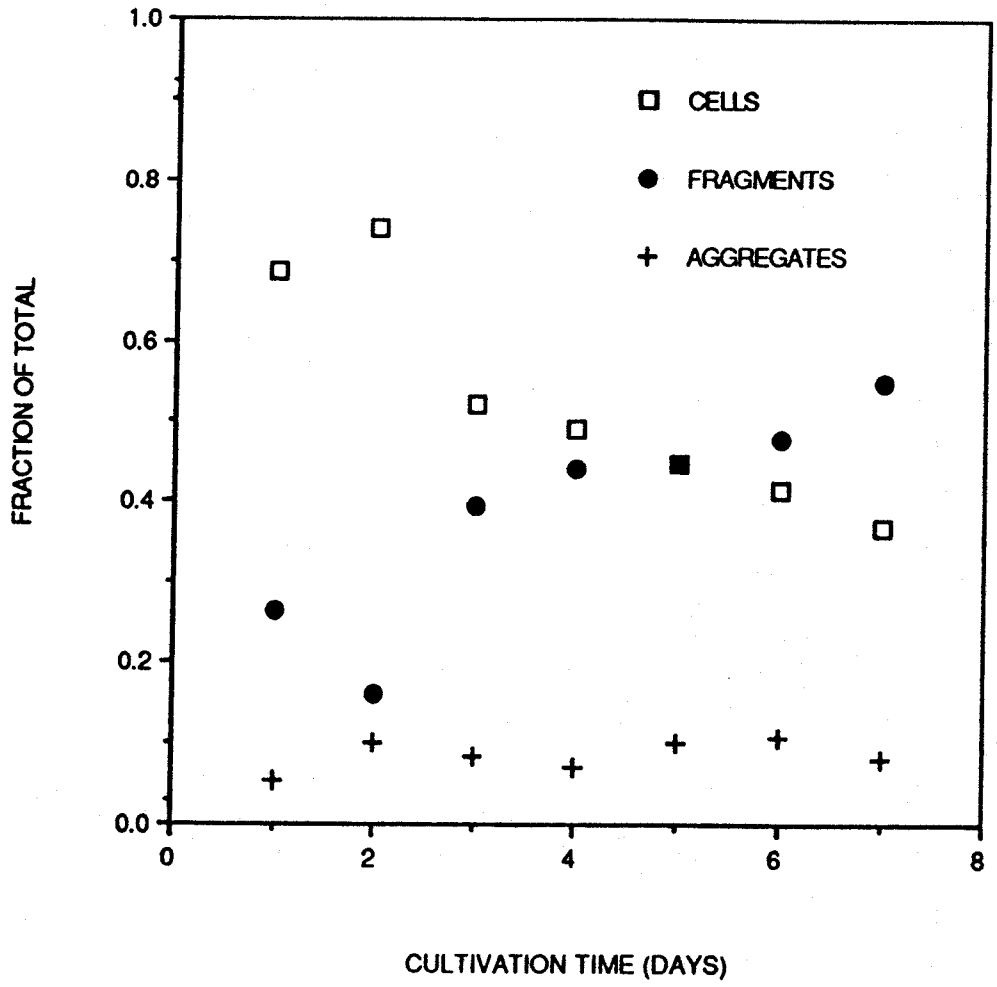

Figure 19b. Bioreactor particle counts based on microscopic analysis, run 2
Cultivation Conditions:

total liquid volume = 1600 mL	150 rpm
inoculum volume = 300 mL	27 °C
age of inoculum = 8 days	paddle impeller
nitrogen flow = 1.14 L/min.	
air flow = 0.7 L/min.	

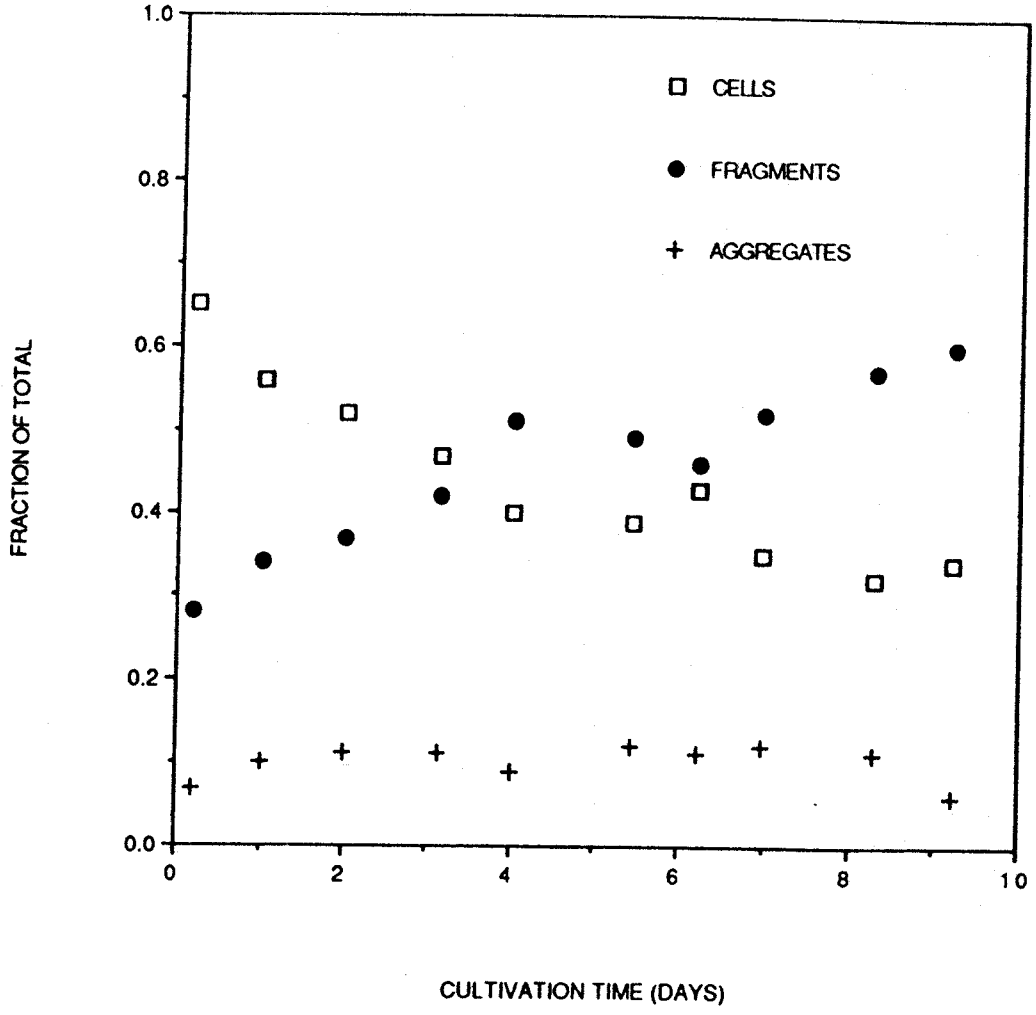

Figure 19c. Bioreactor particle counts based on microscopic analysis, run 3.
Cultivation Conditions:

total liquid volume = 1800 mL	150 rpm
inoculum volume = 500 mL	27 °C
age of inoculum = 8 days	paddle impeller
air flow = 0.8 L/min.	

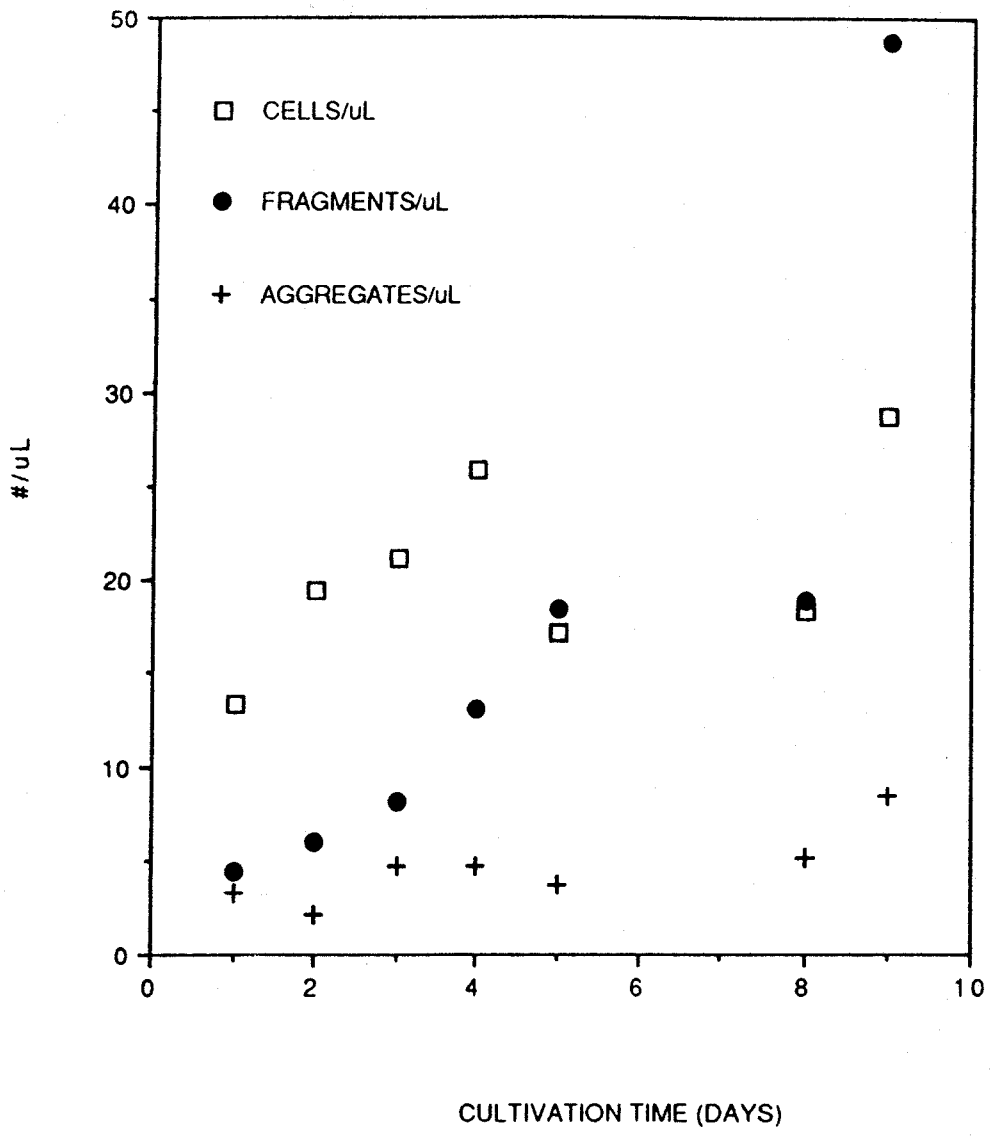

Figure 19d. Bioreactor particle counts on a volume basis based on microscopic analysis, run 1.
Cultivation Conditions:
total liquid volume = 1600 mL
inoculum volume = 300 mL
age of inoculum = 8 days
nitrogen flow = 1.14 L/min.
air flow = 0.7 L/min.
150 rpm
27 °C
paddle impeller

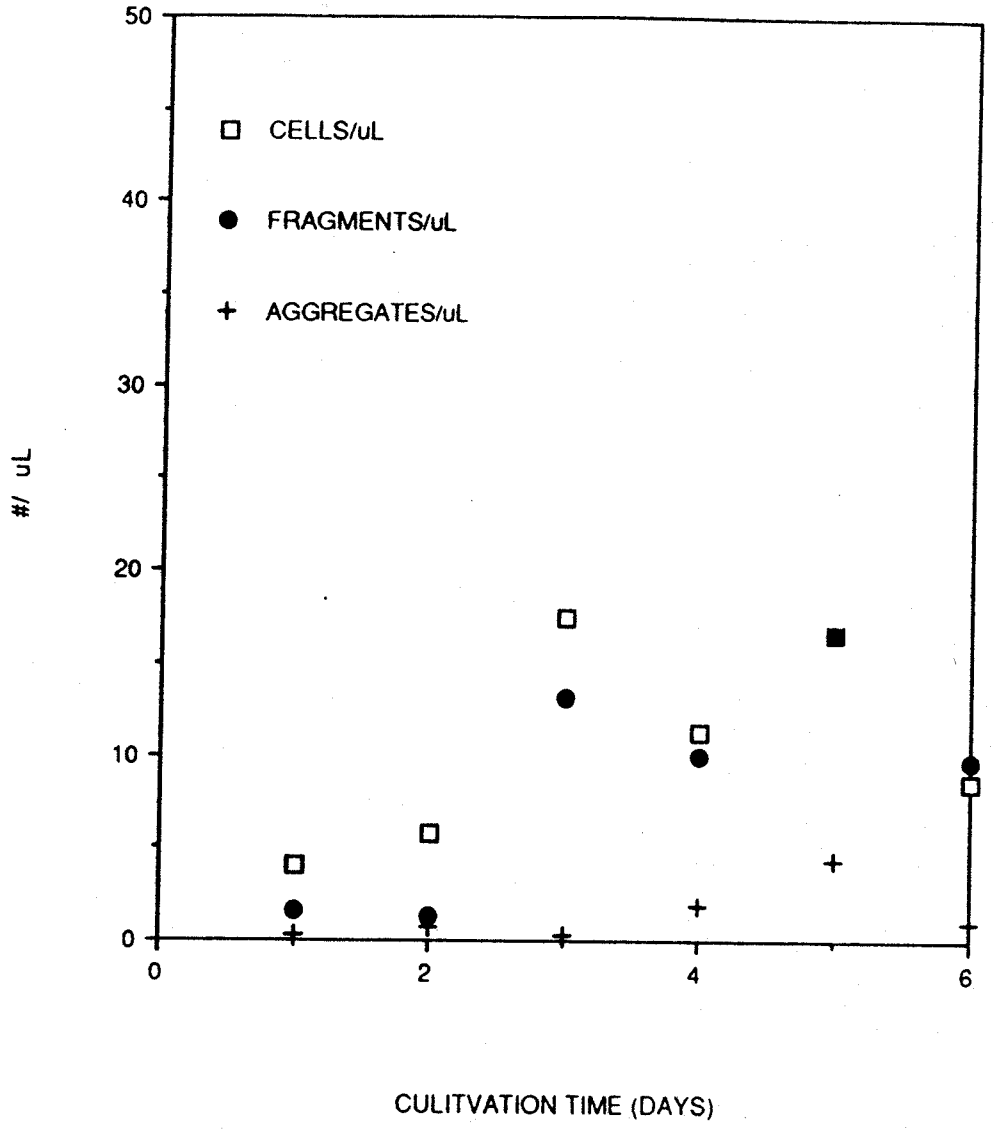

Figure 19e. Bioreactor particle counts on a volume basis based on microscopic analysis, run 2.
Cultivation Conditions:

total liquid volume = 1600 mL	150 rpm
inoculum volume = 300 mL	27 °C
age of inoculum = 8 days	paddle impeller
nitrogen flow = 1.14 L/min.	
air flow = 0.7 L/min.	

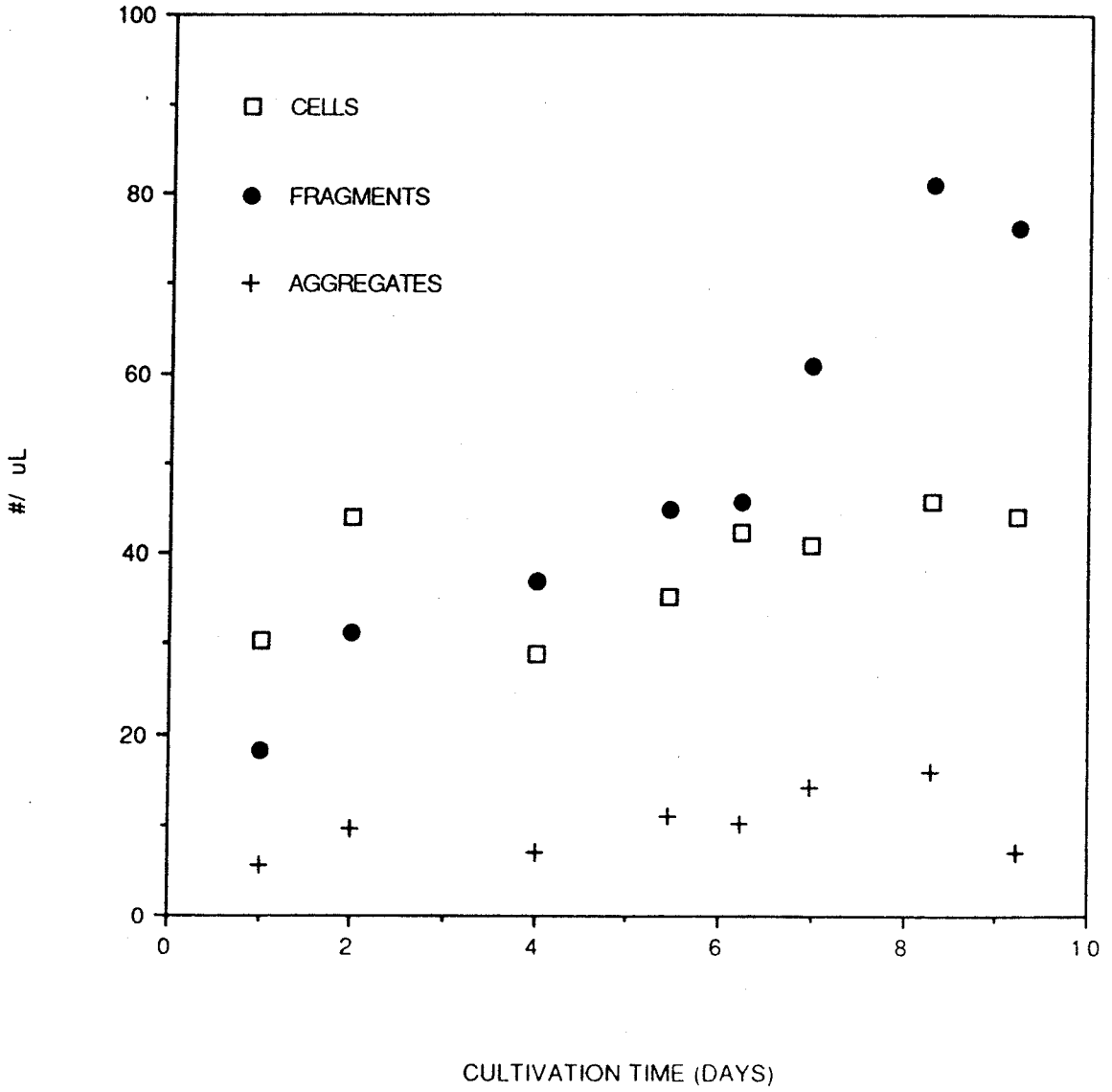

Figure 19f. Bioreactor particle counts on a volume basis based on microscopic analysis, run 3
Cultivation Conditions:

total liquid volume = 1800 mL	150 rpm
inoculum volume = 500 mL	27 °C
age of inoculum = 8 days	paddle impeller
air flow = 0.8 L/min.	

as a function of culture time are shown in Figure 20. In general k_La values decreased slowly until day seven and then increased drastically thereafter. The respiration rate decreased after day three, and then fluctuated around 0.5 mmole O_2/L for the remainder of the cultivation period.

In the second method, no nitrogen was sparged over the surface of the culture. Estimates for k_La and Q_o show a decrease in both k_La and Q_o throughout the cultivation period (Figure 21). The cell cultivation was stopped on day nine because no respiration rate was observed.

The difference in the two sets of curves indicates that surface mixing affects the estimate of both k_La and Q_o. A significant fluctuation in estimates for both k_La and Q_o was noticed in experiments using a nitrogen blanket. The fluctuation is believed to have been caused by ineffective venting of exiting gasses from the bioreactor. As a result, the estimates obtained without sparging nitrogen over the surface are believed to be more accurate.

The model developed to determine k_La as a function of cultivation time showed good agreement with experimental results. An example of a comparison between values predicted by the model and by actual experimental data can be seen in Figure 22. Although the assumptions used in the derivation of the model to predict the D.O. concentration as a function of time can create errors in the estimate for k_La, the exceptional fit of the model to data indicates that the estimate for k_La is adequate.

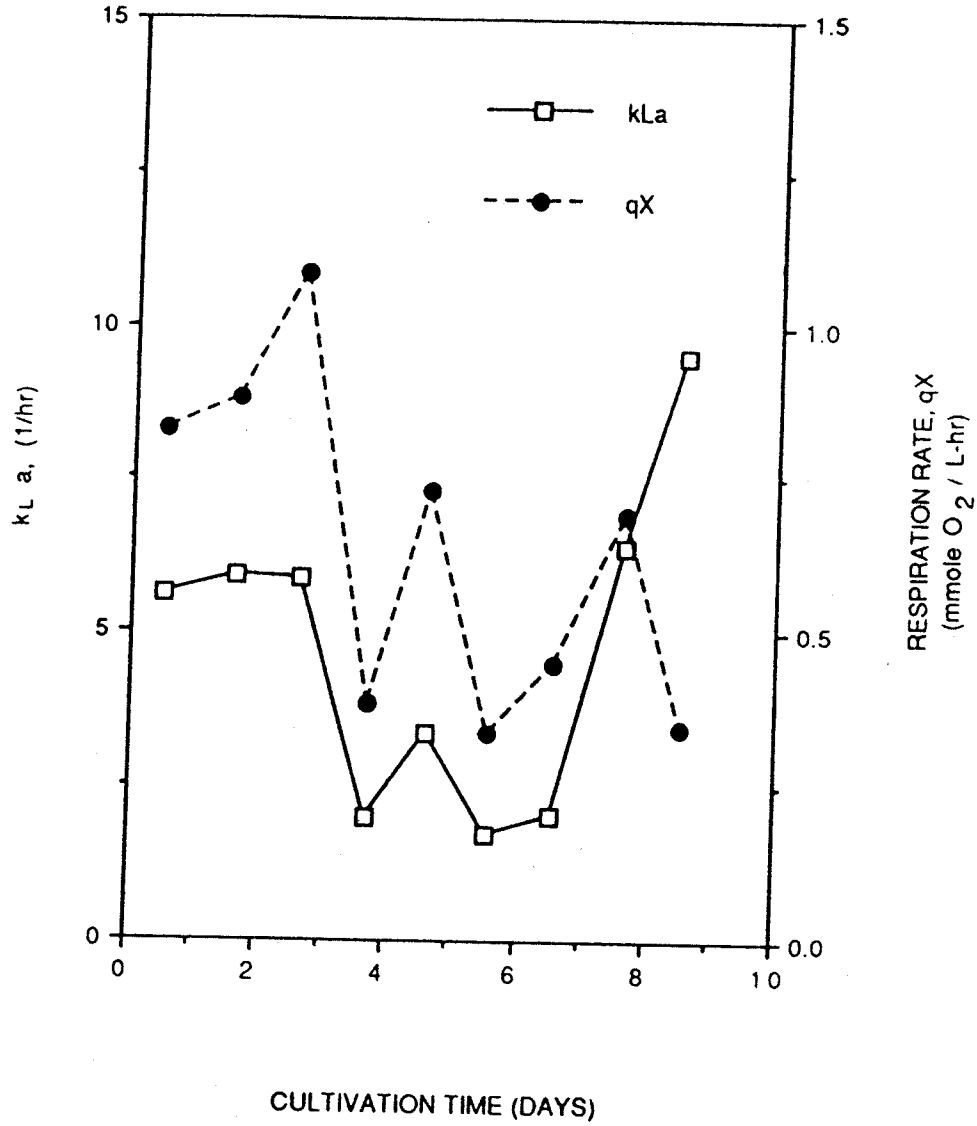

Figure 20a. Estimates of k_La and qX during the cultivation of tobacco cells in the bioreactor while nitrogen was blanketed over the surface, run 1.
Cultivation Conditions:

total liquid volume = 1600 mL	150 rpm
inoculum volume = 300 mL	27 °C
age of inoculum = 8 days	paddle impeller
nitrogen flow = 1.14 L/min.	
air flow = 0.7 L/min.	

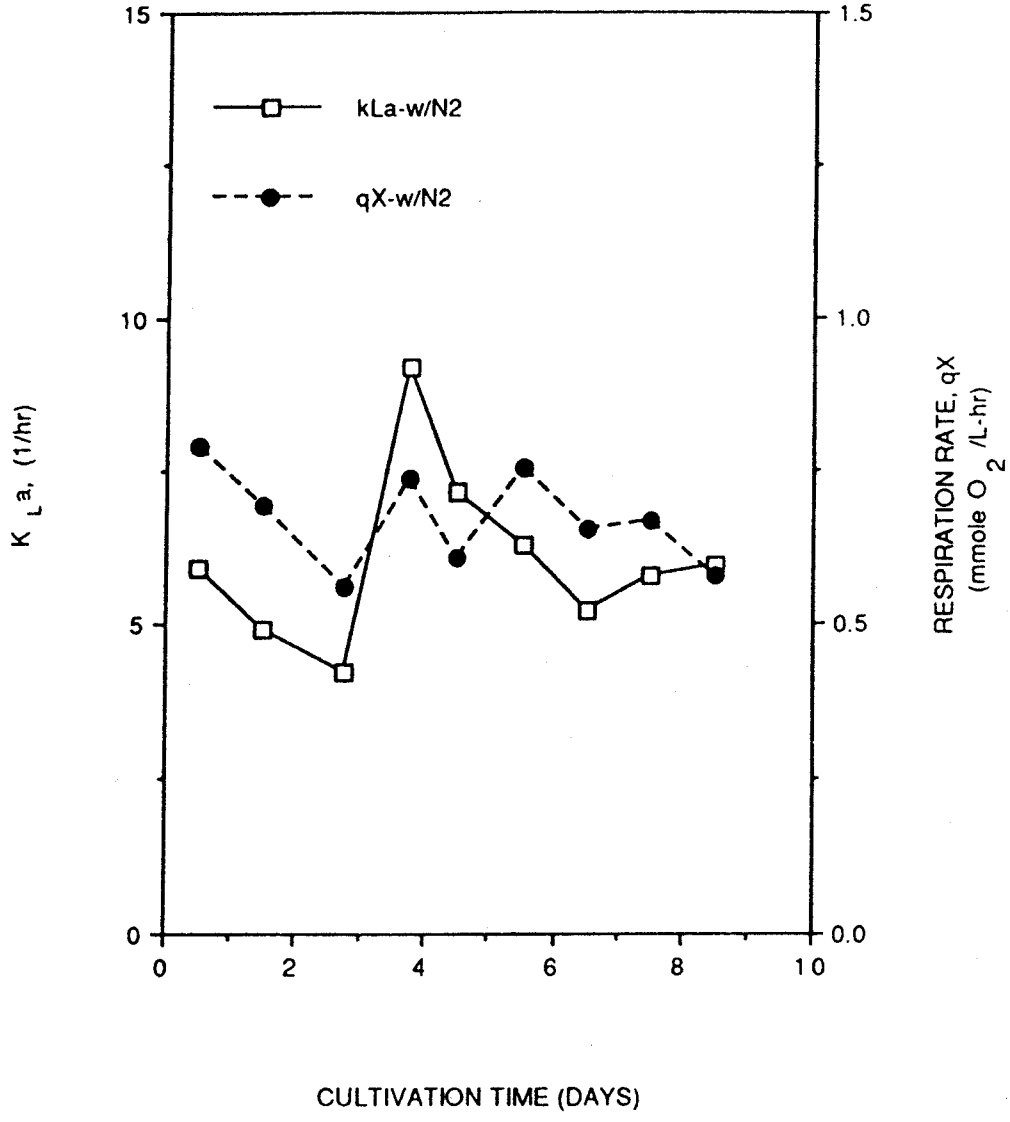

Figure 20b. Estimates of k_La and qX during the cultivation of tobacco cells in the bioreactor while nitrogen was blanketed over the surface, run 2.
Cultivation Conditions:

total liquid volume = 1600 mL	150 rpm
inoculum volume = 300 mL	27 °C
age of inoculum = 8 days	paddle impeller
nitrogen flow = 1.14 L/min.	
air flow = 0.7 L/min.	

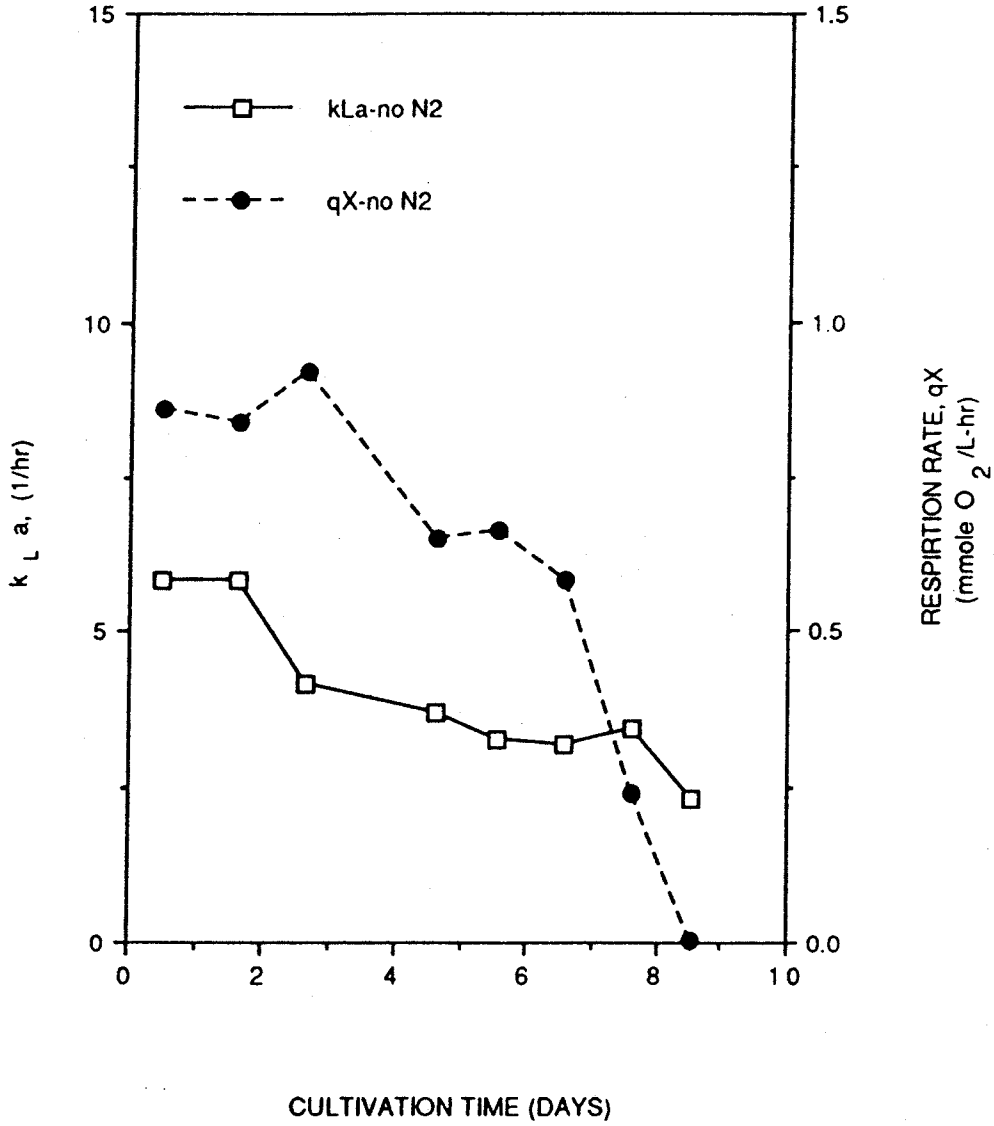

Figure 21a. Estimates of k_La and qX in the bioreactor for the cultivation of tobacco cells with no nitrogen blanket, run 1.
Cultivation Conditions:

total liquid volume = 1600 mL	150 rpm
inoculum volume = 300 mL	27 °C
age of inoculum = 8 days	paddle impeller
nitrogen flow = 1.14 L/min	
air flow = 0.7 L/min.	

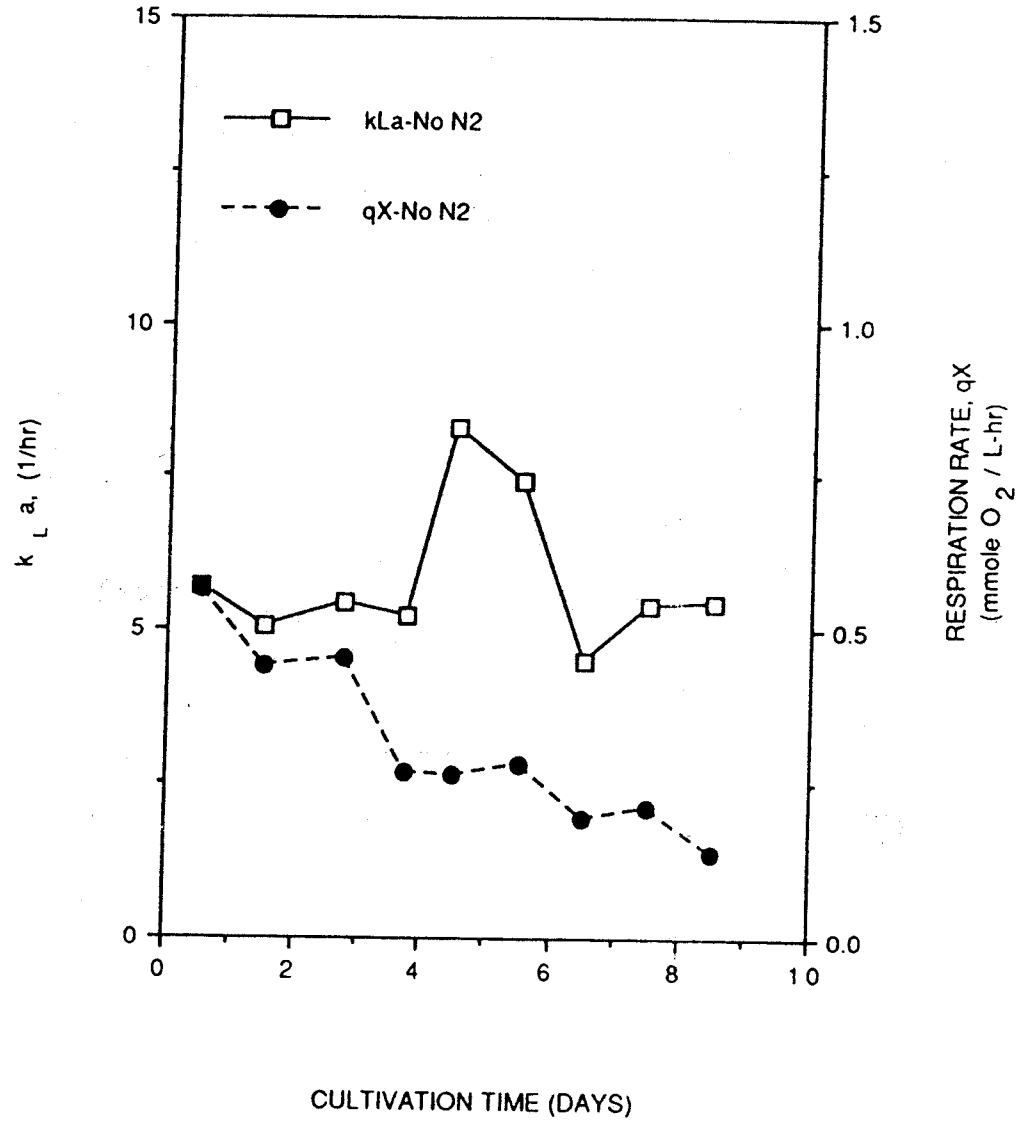

Figure 21b. Estimates of k_La and qX in the bioreactor for the cultivation of tobacco cells with no nitrogen blanket, run 2.
Cultivation Conditions:
total liquid volume = 1600 mL
inoculum volume = 300 mL
age of inoculum = 8 days
nitrogen flow = 1.14 L/min.
air flow = 0.7 L/min.
150 rpm
27 °C
paddle impeller

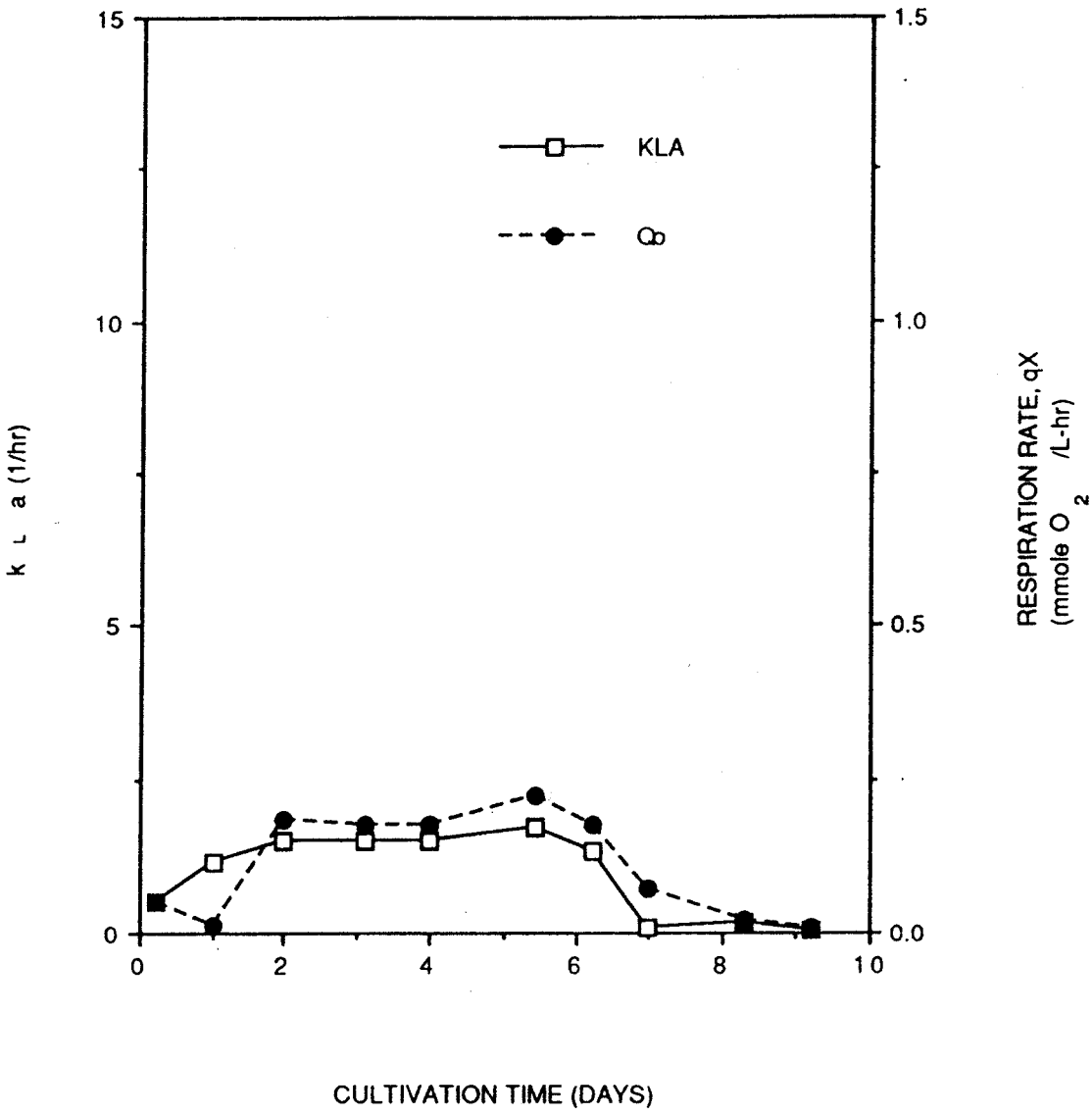

Figure 21c. Estimates of k_La and qX in the bioreactor for the cultivation of tobacco cells with no nitrogen blanket, run 3.
Cultivation Conditions:

total liquid volume = 1800 mL	150 rpm
inoculum volume = 500 mL	27 °C
age of inoculum = 8 days	paddle impeller
air flow = 0.8 L/min.	

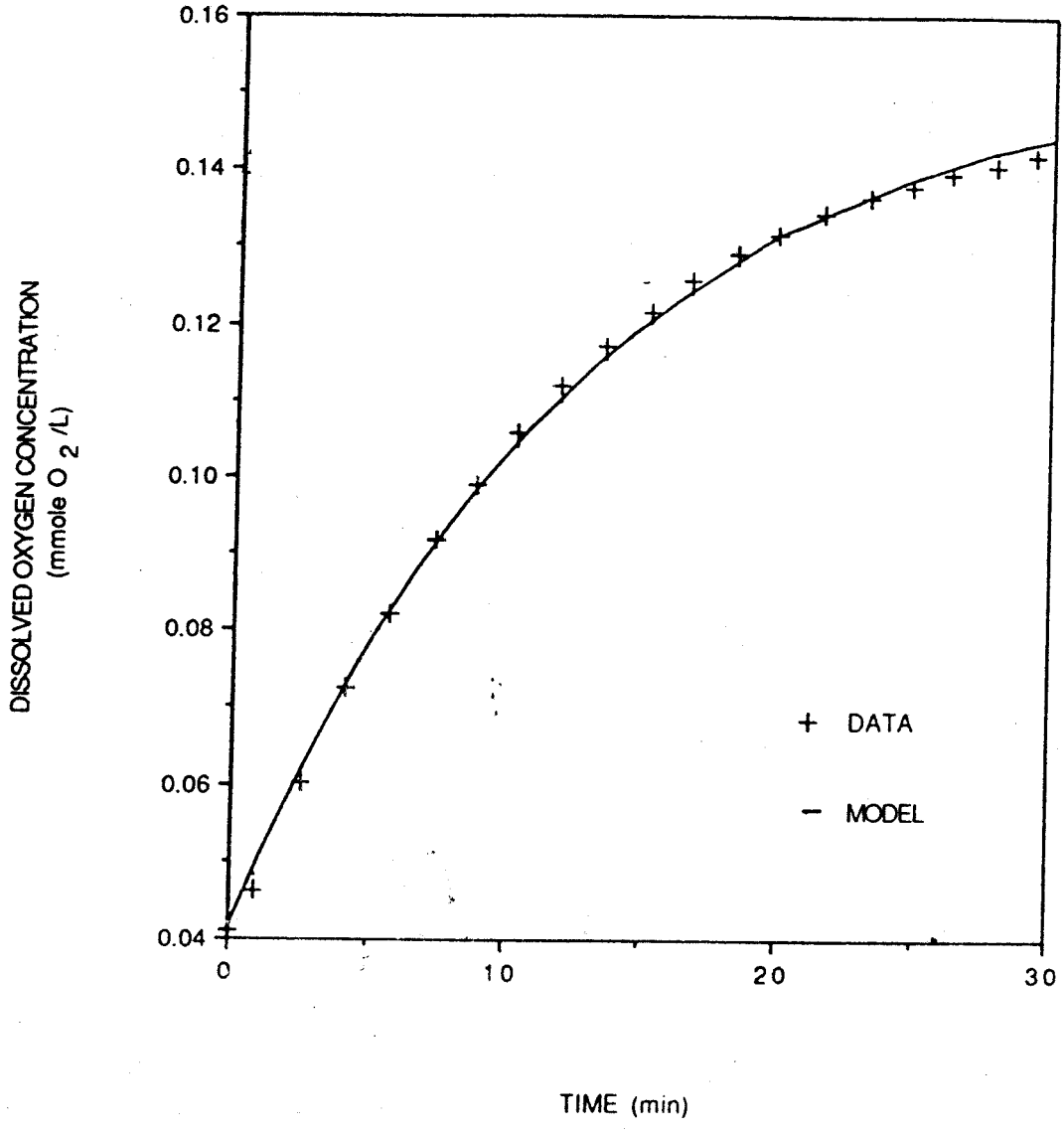

Figure 22. Predicted time dependent oxygen curve and experimentally determined oxygen curve for a dissolve oxygen step change during re-aeration of the culture in the bioreactor.
Cultivation Conditions:

total liquid volume = 1600 mL	150 rpm
inoculum volume = 300 mL	27 °C
age of inoculum = 8 days	paddle impeller
nitrogen flow = 1.14 L/min.	
air flow = 0.7 L/min.	

Therefore, this method for *in situ* estimation of k_La in an aerobic plant cell culture bioreactor proved satisfactory.

Sugar Utilization, pH and % D.O. Measurements

Also measured during the cultivation of tobacco cells in the bioreactor were pH, %D.O., and sugar utilization. The pH held steady at about 5.4 ± 0.2 until day eight when it dropped drastically to 4.6 as can be seen in Figure 23. The dissolved oxygen concentration decreased steadily during the cultivation until day eight and then it increased dramatically (Figure 24). The significant decrease in both pH and %D.O. correspond to the decrease observed in Q_o for the estimates when nitrogen was not sparged over the surface. The time for the decreases also corresponds to the maximum volume of cell fragments. The combination of all four trends indicates a significant increase in cell deformation and death.

The sugar utilization was similar to what occurred in the shake flask studies. The sucrose was hydrolyzed into fructose and glucose. Sucrose, glucose, and fructose utilization curves can be seen in Figure 25. The total sugar utilization curve can be seen in Figure 26. At the end of the bioreactor cultivation period, the total sugar level in the culture medium was 10.3 g/L and 10.0 g/L for bioreactor runs 1 and 2 respectively.

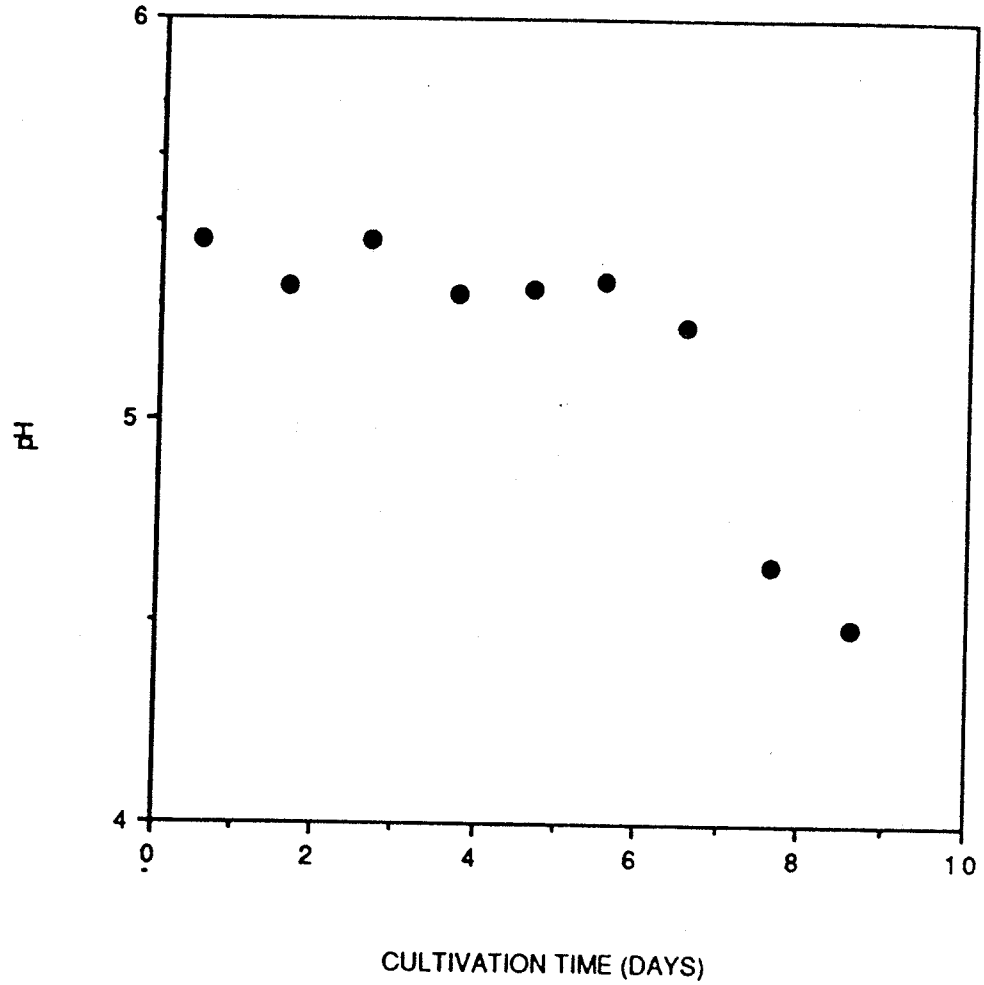

Figure 23a. Measurements of pH for cultivation of tobacco cells in bioreactor studies, run 1.
Cultivation Conditions:

total liquid volume = 1600 mL	150 rpm
inoculum volume = 300 mL	27 °C
age of inoculum = 8 days	paddle impeller
nitrogen flow = 1.14 L/min.	
air flow = 0.7 L/min.	

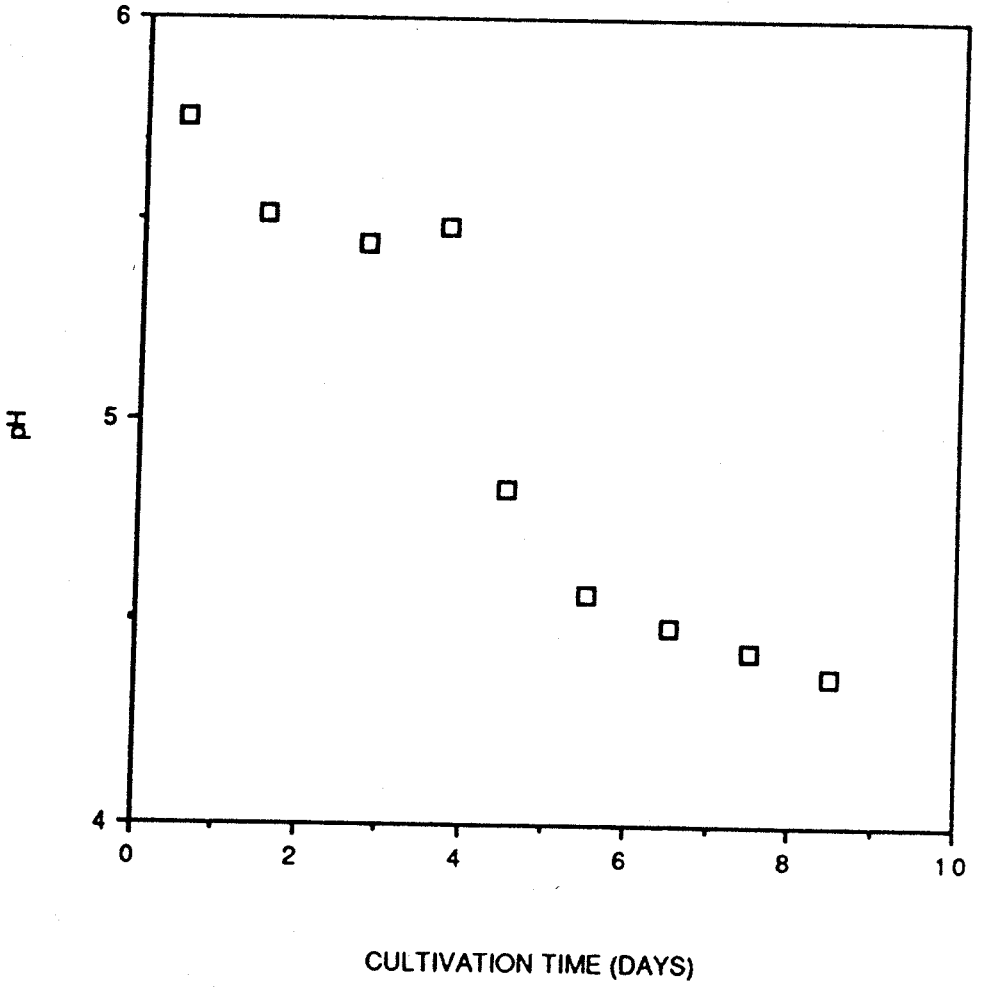

Figure 23b. Measurements of pH for cultivation of tobacco cellsin bioreactor studies, run 2.
Cultivation Conditions:
total liquid volume = 1600 mL 150 rpm
inoculum volume = 300 mL 27 °C
age of inoculum = 8 days paddle impeller
nitrogen flow = 1.14 L/min.
air flow = 0.7 L/min.

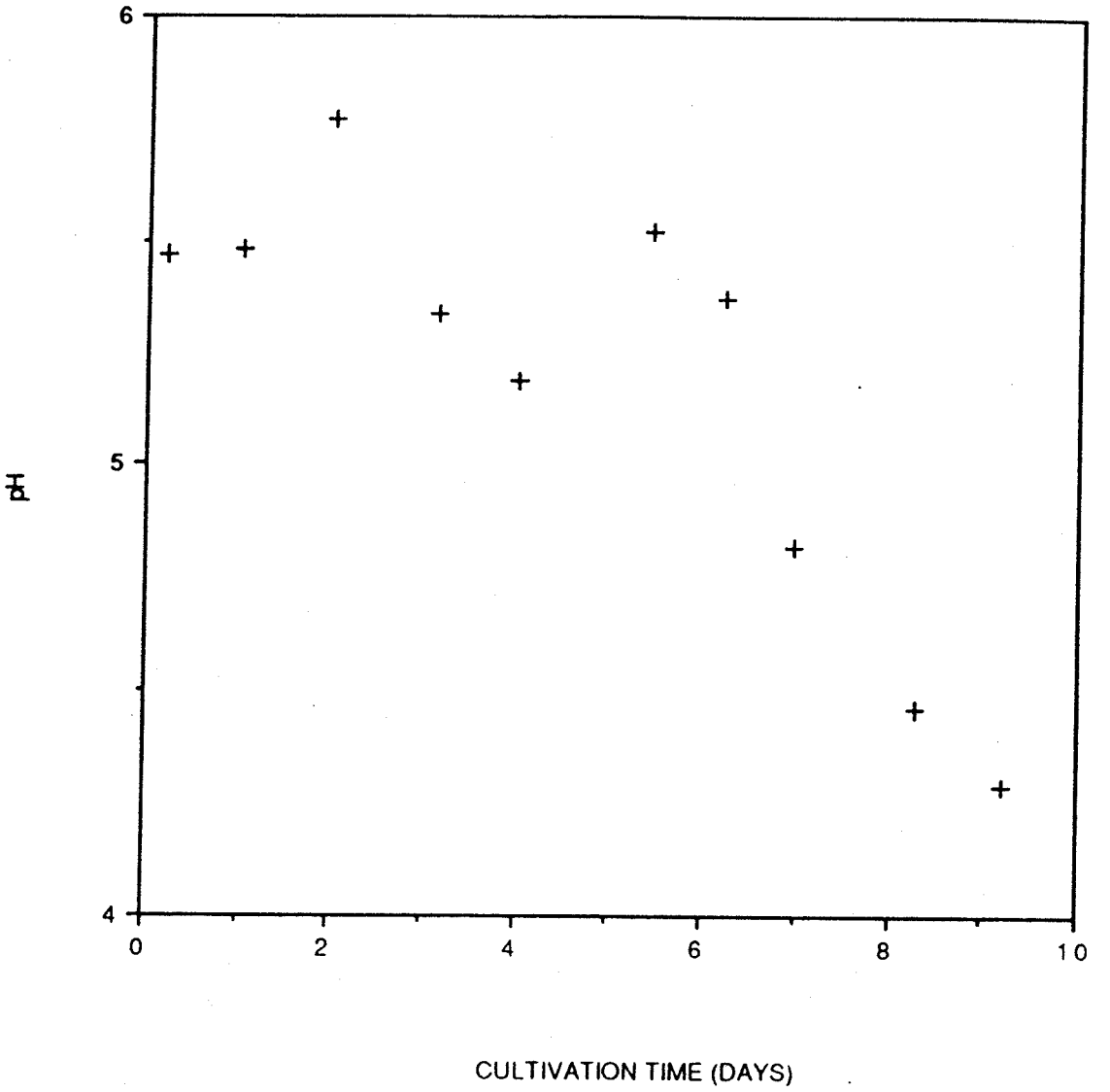

Figure 23c. Measurements of pH for cultivation of tobacco cellsin bioreactor studies, run 3.
Cultivation Conditions:
total liquid volume = 1800 mL 150 rpm
inoculum volume = 500 mL. 27 °C
age of inoculum = 8 days paddle impeller
air flow = 0.8 L/min.

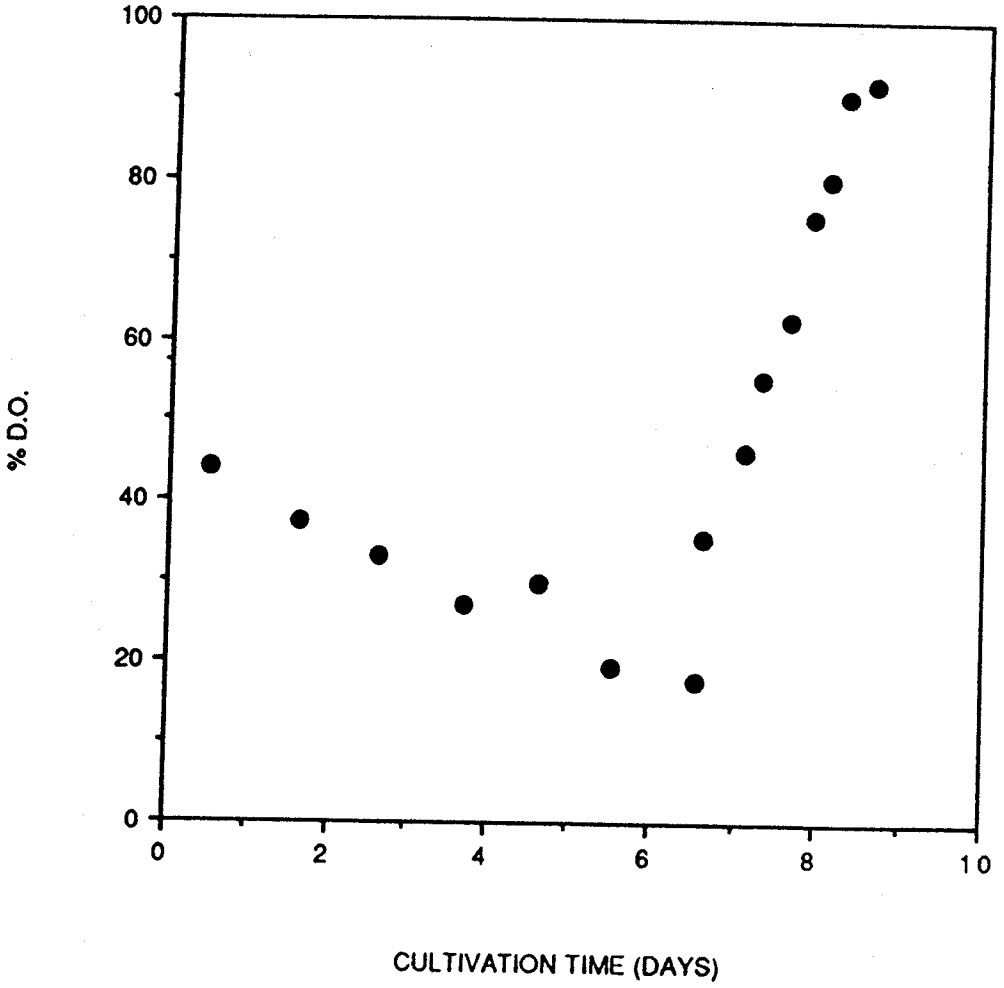

Figure 24a. Dissolved oxygen concentration in bioreactor, expressed as % of saturation with respect to air in medium for cultivation of tobacco cells, run 1.
Cultivation Conditions:

total liquid volume = 1600 mL	150 rpm
inoculum volume = 300 mL	27 °C
age of inoculum = 8 days	paddle impeller
nitrogen flow = 1.14 L/min.	
air flow = 0.7 L/min.	

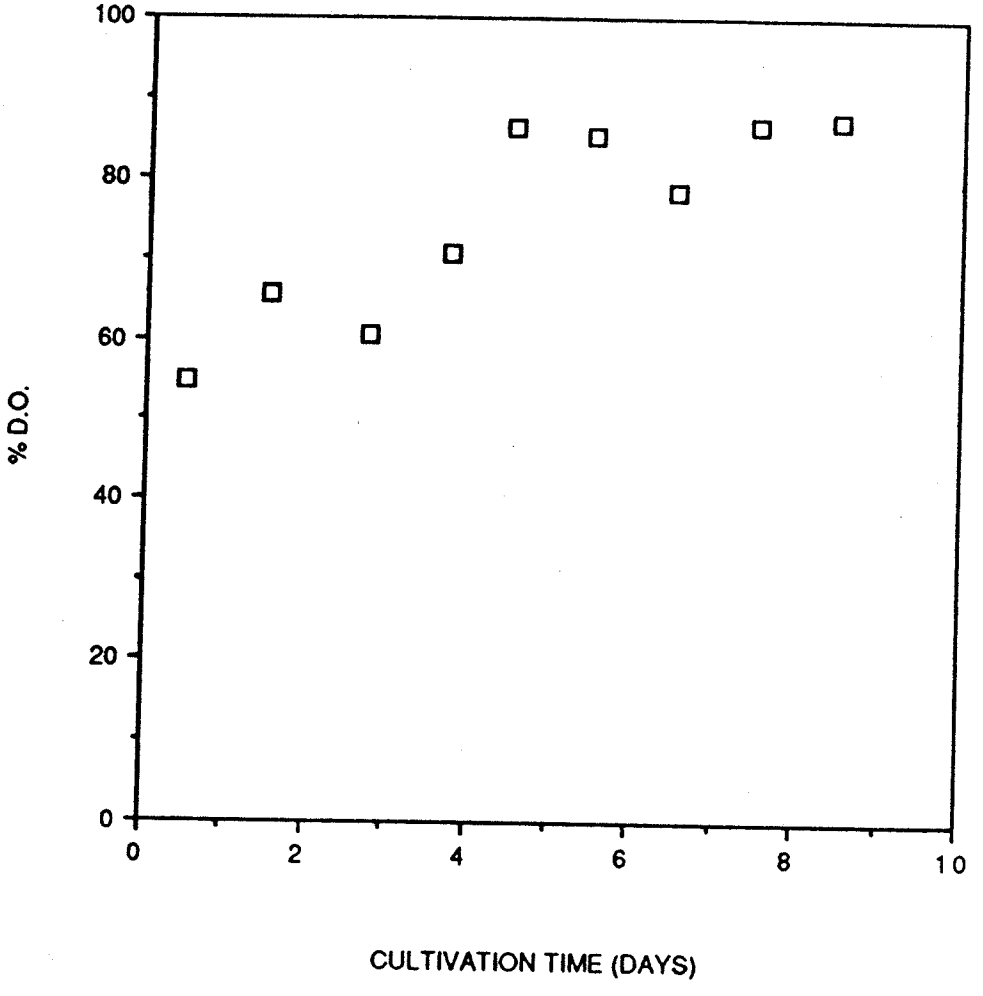

Figure 24b. Dissolved oxygen concentration in bioreactor, expressed as % of saturation with respect to air in medium for cultivation of tobacco cells, run 2.
Cultivation Conditions:

total liquid volume = 1600 mL	150 rpm
inoculum volume = 300 mL	27 °C
age of inoculum = 8 days	paddle impeller
nitrogen flow = 1.14 L/min.	
air flow = 0.7 L/min.	

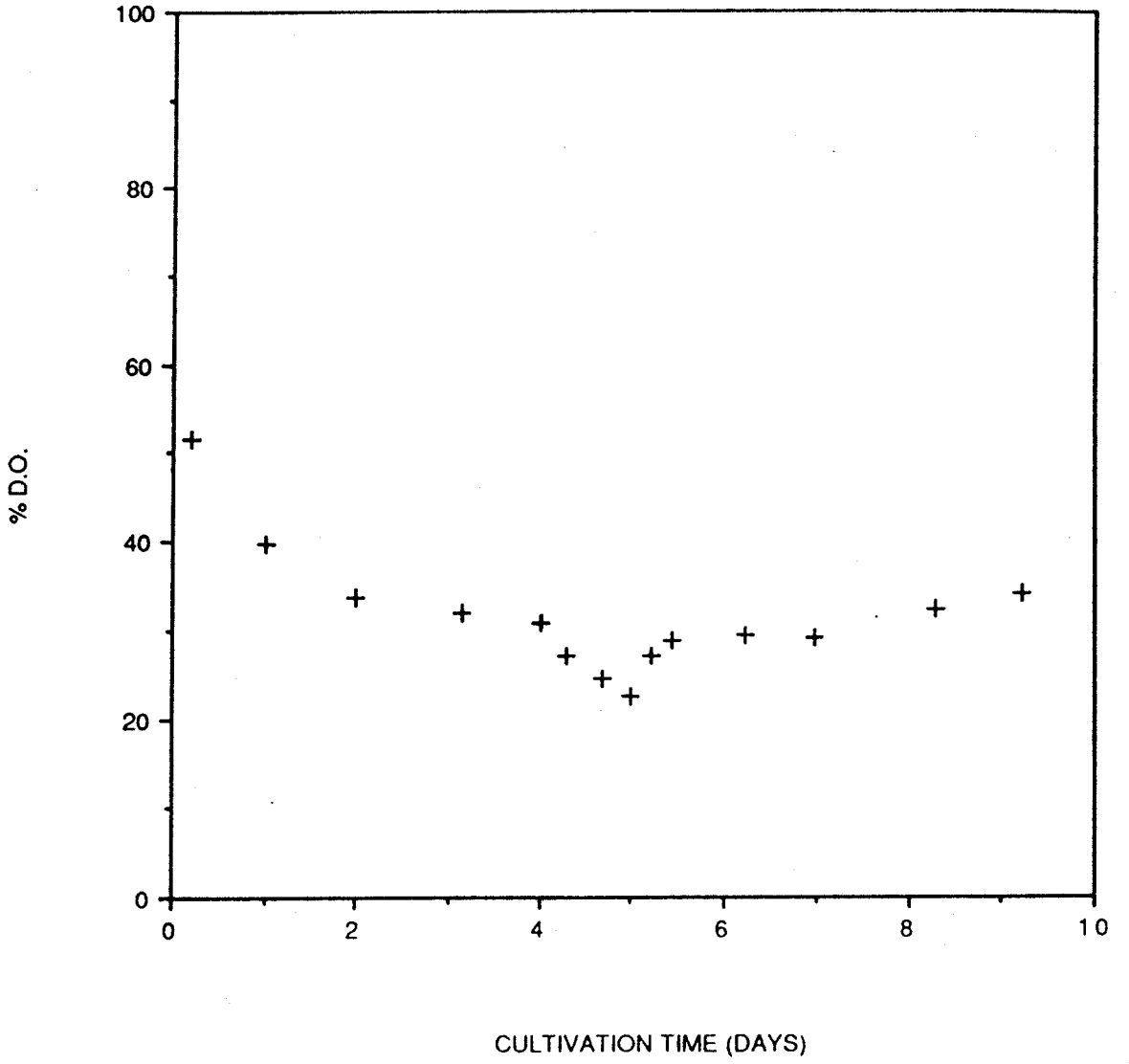

Figure 24c. Dissolved oxygen concentration in bioreactor, expressed as % of saturation with respect to air in medium for cultivation of tobacco cells, run 3.
Cultivation Conditions:

total liquid volume = 1800 mL	150 rpm
inoculum volume = 500 mL	27 °C
age of inoculum = 8 days	paddle impeller
air flow = 0.8 L/min.	

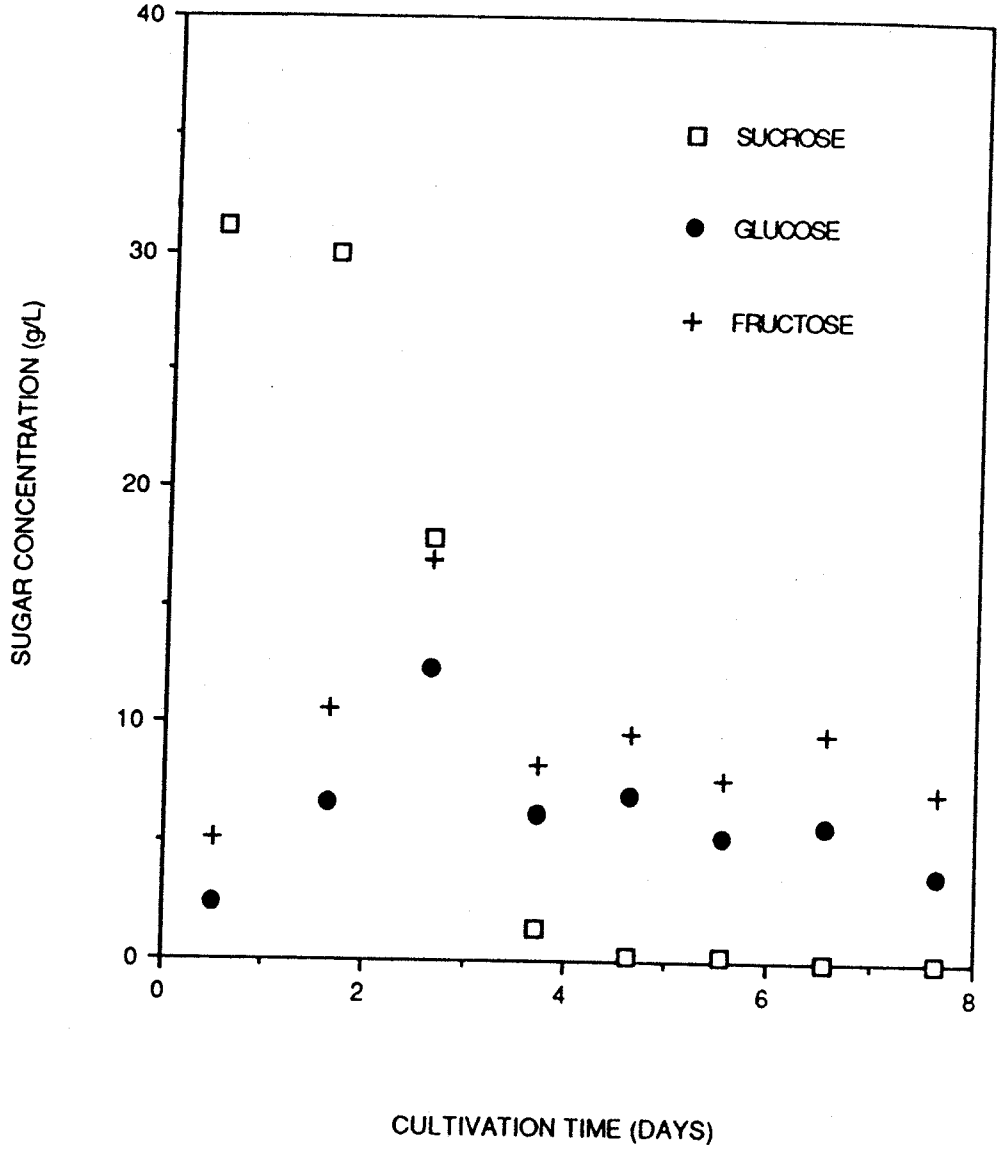

Figure 25a. Sugar utilization curves for bioreactor run 1.
Cultivation Conditions:
total liquid volume = 1600 mL 150 rpm
inoculum volume = 300 mL 27 °C
age of inoculum = 8 days paddle impeller
nitrogen flow = 1.14 L/min.
air flow = 0.7 L/min.

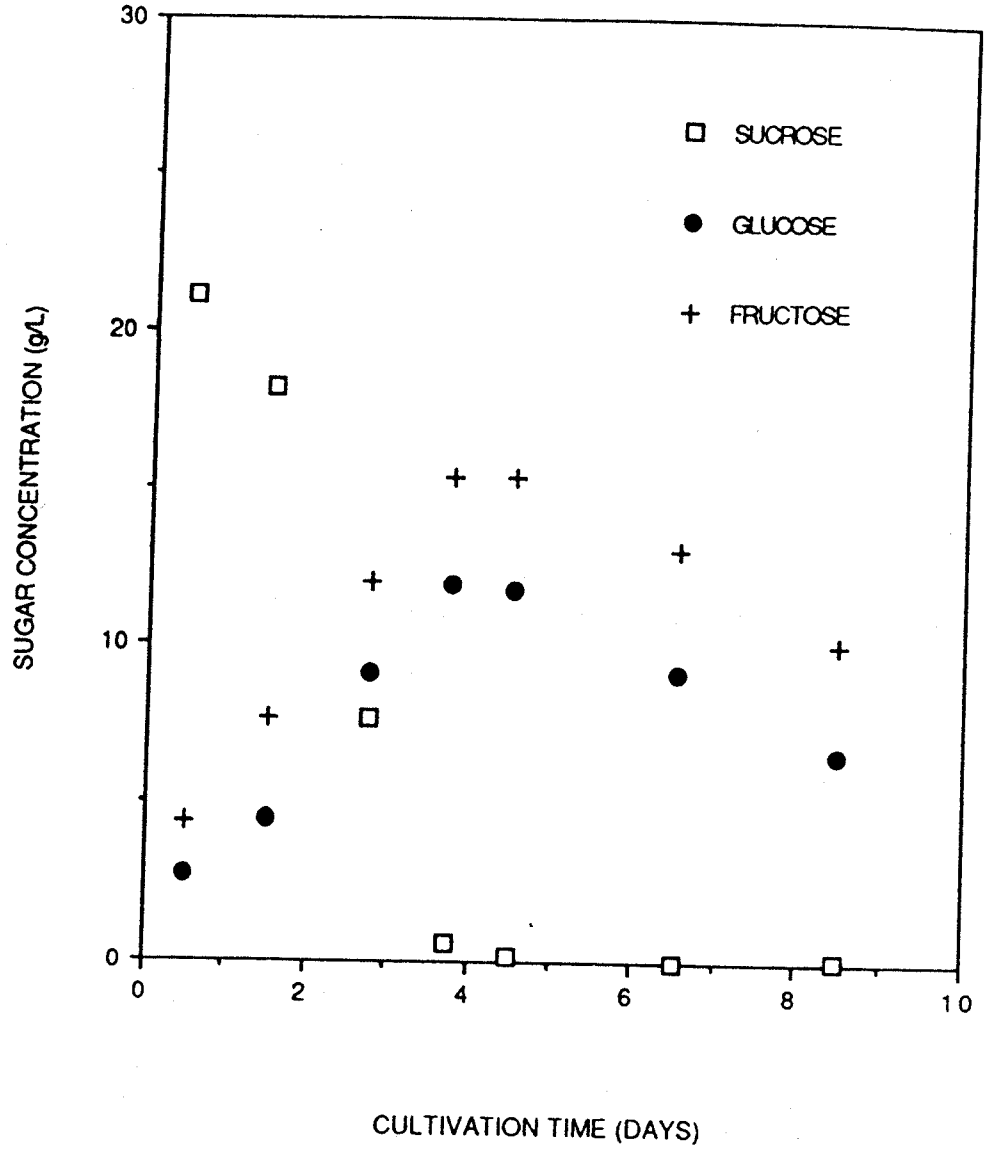

Figure 25b. Sugar utilization curves for bioreactor run 2.
Cultivation Conditions:

total liquid volume = 1600 mL	150 rpm
inoculum volume = 300 mL	27 °C
age of inoculum = 8 days	paddle impeller
nitrogen flow = 1.14 L/min.	
air flow = 0.7 L/min.	

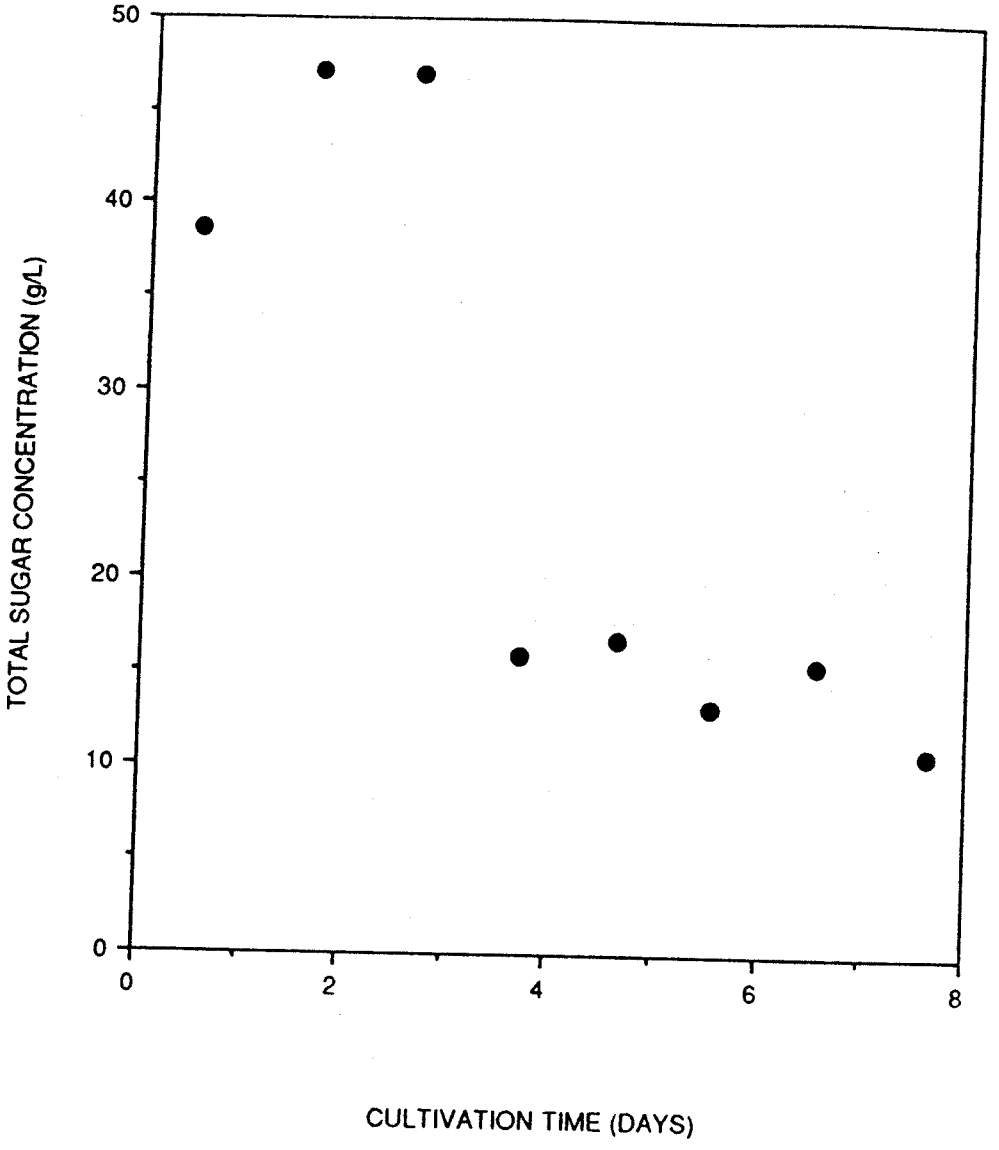

Figure 26a. Toatal sugar utilization curve for bioreactor run 1.
Cultivation Conditions:

total liquid volume = 1600 mL	150 rpm
inoculum volume = 300 mL	27 °C
age of inoculum = 8 days	paddle impeller
nitrogen flow = 1.14 L/min.	
air flow = 0.7 L/min.	

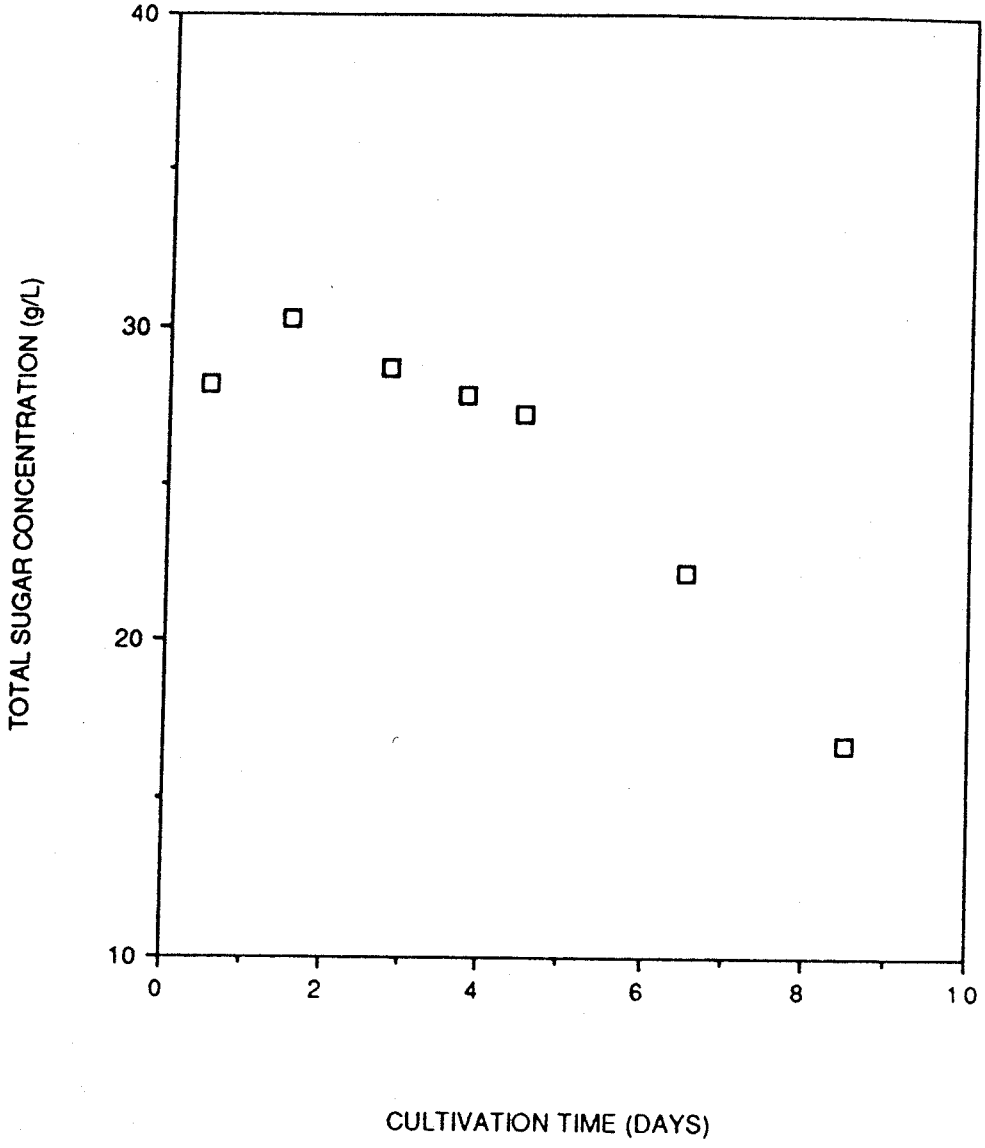

Figure 26b. Toatal sugar utilization curve for bioreactor run 2.
Cultivation Conditions:

total liquid volume = 1600 mL	150 rpm
inoculum volume = 300 mL	27 °C
age of inoculum = 8 days	paddle impeller
nitrogen flow = 1.14 L/min.	
air flow = 0.7 L/min.	

CONCLUSIONS

Suspension cultures of *N. tabacum* var. Wisconsin 38 were grown in both shake flasks and a 2 L stirred-tank bioreactor. The biomass and sugar concentrations were measured as a function of cultivation time for both batch cultivation processes. In addition, the respiration rate and the volumetric mass transfer coefficient were estimated as a function of cultivation time and cell concentration for the bioreactor cultivation studies.

Batch cultures of tobacco cells grown in the 2 L bioreactor using a paddle blade impeller at 27 °C, 0.4 vvm, and 150 rpm showed a much lower growth rate and maximum cell density than in the shake flask control studies. The low growth rate observed in the bioreactor studies is believed to have been caused by the high shear generated by the bioreactor agitation system. Microscopic analysis revealed a sharp increase in cell fragments and a decrease in single cells at the onset of the exponential growth phase. Biomass also decreased significantly during the early exponential growth phase. The microscopic study and biomass measurements indicate both cell rupture and death to have occurred during the exponential growth phase. Sugar utilization studies indicated that insufficient carbon source was not the cause for the cell death.

Although the literature review showed that stirred-tank cultivation of plant cells is feasible, not all cell lines can be

cultivated. For example, Tal et al. [1983] reported that *Discorea* cells were sensitive to the impeller agitation system in a stirred-tank bioreactor during the exponential growth phase and could only be cultivated in a bubble-type fermenter. Another report by Kim [1989] indicated that *Thalictrum* suspension cultures could not be grown in a stirred-tank reactor, yet could be cultivated in an air-lift reactor [Kim et al, 1991].

Coupled with the decrease in both cell biomass and single cells during the exponential growth phase was the decrease in pH, increase in the dissolved oxygen level, and decrease in the respiration rate of the cells. All of these phenomena indicate that culture growth was not being sustained.

The model developed for *in situ* estimates of k_La as a function of cultivation time and cell concentration proved to work adequately. Many assumptions were incorporated into the model. However, the fit of the model to dissolved oxygen concentration versus time data was excellent. It is felt that this model is sufficient for *in situ* estimation of k_La. Once k_La is determined, this parameter can be used to study the effect of bioreactor process variables and bioreactor design on oxygen mass transfer, cell growth, and shear sensitivity in stirred-tank plant cell cultivation.

The unique contribution of this study are :

1. *Nicotiana tabacum* var. Wisconsin 38 was characterized through shake flask studies.

2. Sensitivity of plant cells to mechanicalagitation was supported by:
   a. microscopic analysis
   b. *in situ* respiration rate estimates
   c. *in situ* pH and % D.O. measurements
   d. biomass measurements

3. The mass transfer coefficient was estimated as a function of biomass and cultivation time. No reported work was uncovered in which *in situ* estimates of k_La had been previously performed.

RECOMMENDATIONS

The cultivation of *N. tabacum* var. Wisconsin 38 in the 2 L stirred-tank bioreactor did not reach the high cell densities seen in the shake flask studies. Both an optimization study on bioreactor process conditions and design alterations of the bioreactor itself are recommended with the hopes of increasing cell density. The optimization study involves finding the optimal settings for both aeration and agitation rates, and ideal initial sucrose concentration in the medium. While literature was thoroughly investigated to come up with the best possible settings for the stirred-tank bioreactor, it is felt that the process parameters could be improved upon with actual experimental data.

The bioreactor design should be altered to decrease the amount of shear that the culture is subjected to. The agitation system in the reactor is magnetically driven. The magnet for the system is located in the bioreactor and occupies a total volume of 45 cm^3. The rotation of the large magnet adds unnecessary shear to the system. A mechanically driven agitation system with the rotor located on the outside of the bioreactor where only the shaft and impeller contact the culture could lessen the hydrodynamic shear.

Another possibility for increasing the cell density in the bioreactor and decreasing the hydrodynamic shear would be to change the agitation rate when the culture enters the exponential

growth phase. The lowed agitation rate could decrease the shear during the period in which the culture is most susceptible to shear damage.

The variety of tobacco cells used in this study were shear sensitive. It is recommended to cultivate a less shear sensitive variety of tobacco cells, e.g. Bright Yellow, which have already been cultivated successfully in previous reported investigations.

After optimizing the bioreactor process conditions and successfully cultivating cells at high density, it is recommended to alter the process variables. The alterations could offer insight on how these variables effect k_La and thus gas transport characteristics. Also recommended would be to scale-up the bioreactor to 10 L and investigate the effectiveness of k_La as a means to predict biomass growth for plant cell cultivation in a stirred-tank bioreactor.

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APPENDICES

APPENDIX A: COMPUTER PROGRAMS.

A FORTRAN computer program is listed which was used to calculate both the respiration rate (qX) and the volumetric mass transfer coefficient (k_La). A previously created data file consisting of concentration versus time data for a pulse test on the aeration in the bioreactor is opened. The respiration rate is first calculated using a least squares linear regression method on the linear de-gassing portion of the concentration versus time data. The Gauss-Newton nonlinear regression analysis is then used to determine k_La from the portion of the concentration versus time data in which the bioreator is being re-aerated (increasing concentration) and the calculated respiration rate.

```
      PROGRAM BIO
*
* *   THIS PROGRAM USES THE GAUSS-MINIMIZATION
* *   TECHNIQUE TO DETERMINE KLA. DATA FROM A
* *   BIOREACTOR RESPIRATION PULSE-TEST WILL
* *   BE READ, AND KLA DETERMINED
*
      DIMENSION  T(1000),C(1000),CM(1000),DCM(1000)
      DIMENSION  S(1000),CT(1000),TT(1000)
      REAL KLA, KLAP
      CHARACTER*15 RUN
      OPEN(UNIT=10,FILE='BIO.DAT',STATUS='OLD')
      OPEN(UNIT=11,FILE='RESP.DAT',STATUS='NEW')
      OPEN(UNIT=12,FILE='DKLA.DAT',STATUS='NEW')
      OPEN(UNIT=13,FILE='MODEL.DAT',STATUS='NEW')
*
* *   READ IN DATA FROM BIOREACTOR RUN, AND SET TO=0
*
      PRINT*,'ENTER IN BIOREACTOR RUN NUMBER'
      READ*,RUN
      EPS=0.005
      SUMT=0.0
      SUMC=0.0
      SUMCT=0.0
      SUMT2=0.0
      COUNT=0.0
      N=1
5     READ(10,*,END=100) T(N),C(N)
      N=N+1
      GO TO 5
100   DO 10 J=4,N-1
        IF(C(J-3).GT.C(J-2)) THEN
          IF(C(J-2).GT.C(J-1))THEN
            IF (C(J-1).GT.C(J)) THEN
            K= J-3
            COUNT=COUNT+1
              WRITE (11,*) C(J-3),T(J-3)
            CT(J-3)=C(J-3)*T(J-3)
            TT(J-3)=T(J-3)**2
            SUMC=SUMC+C(J-3)
```

```
                SUMT=SUMT+T(J-3)
                SUMCT=SUMCT+CT(J-3)
                SUMT2=SUMT2+TT(J-3)
                ENDIF
              ENDIF
            ENDIF
 10     CONTINUE
        BB=(SUMCT-SUMT*SUMC/COUNT)/(SUMT2-SUMT**2/COUNT)
        QX=ABS(BB*8.6/100/32*3600)
        PRINT*,'QX=',QX
        DO 20 M=K+2,N-1
              WRITE(12,*)  C(M),T(M)
 20     CONTINUE
        T0=T(K+2)
        C0=C(K+2)*8.6/100/32
        DO 30  LL=K+3,N-1
             T(LL)=(T(LL)-T0)/3600
             C(LL)=(C(LL)*8.6/100)/32
 30     CONTINUE
*
* *    CALCULATE VALUES FOR CM(N) AND DCM(N) USING T(N).
* *    ANALYTICAL INTEGRATION PROVIDES THE TWO EQUATIONS
*
        PRINT *,'ENTER IN VALUE FOR CSAT'
        READ*,CSAT
        PRINT*,'ENTER IN INTITAL GUESS FOR KLA'
        READ*,KLA
        ITER=0
        DO 50 JJ=1,50
             DO 40 I=K+3,N-1
               A=CSAT - QX/KLA
                 B=QX/KLA**2*(1-EXP(-KLA*T(I)))
                CM(I)=A-(A-C0)*EXP(-KLA*T(I))
                DCM(I)=(C0-A)*(-T(I))*EXP(-KLA*T(I))+B
               S(I)= C(I)-CM(I)
 40          CONTINUE

* *    GAUS-MINIMIZATION STEP
*
             E=0
             F=0
             SR=0
```

```
            DO 60 L=K+3,N-1
                  E=E + DCM(L)*S(L)
                  F=F + DCM(L)**2
                  SR=SR+S(L)**2
 60         CONTINUE
      KLAP = KLA+E/F
      CON= ABS((KLAP - KLA)/KLAP)
      ITER=ITER+1
      IF (CON.LT.EPS) THEN
        GO TO 200
      ENDIF
      KLA=KLAP
 50   CONTINUE
 200  DO 65 MM=K+3,N-1
            WRITE(13,*) C(MM),CM(MM),T(MM)
 65   CONTINUE
*
* *   KLA HAS BEEN DETERMINED AND A SUM OF THE SQUARES
* *   OF THE RESIDUAL CALCULATED.
*
      PRINT 220,RUN
      PRINT 230,ITER
      PRINT 300,KLAP
      PRINT 400,SR
 220  FORMAT(//1X,'BIOREACTOR RUN: ',A)
 230  FORMAT(1X,'NUMBER OF ITERATIONS= ',I3)
 300  FORMAT(/1X,'KLA= ',F8.5)
 400  FORMAT(1X,'THE SUM OF THE SQUARE OF THE RESID = ',F8.5)
      END
```

A BASIC computer program, written by Vasja Marjanovic, is listed below. An analog output from the VirTis dissolved oxygen meter was sent the computer. The BASIC program used PCLAB subroutines. The program processed and stored the incoming concentration versus time data and plotted it out in real time onto the CRT display.

```
800 'O2%:  by Vasja Marjanovic
810 'PCLAB SUBROUTINES: MEASURE.VOLTS, ERROR ROUTINES
905 GOSUB 5000
910 KEY OFF
912 ON KEY(3) GOSUB 8000
920 CLS
930 PRINT "O2%:      This program takes data from a dissolved
oxygen      meter (at "
940 PRINT "          ten second intervals) on channel 2, writes it to
a data"
950 PRINT "          file you choose, and plots it.  All in real time."
955 PRINT
960 PRINT "Plot for:  (3)0 OR (6)0 MINUTES?  WILL SAMPLE UNTIL
YOU  BREAK.":ST$=INPUT$(1):IF ST$<>"3" AND ST$<>"6" THEN 960
962 IF ST$="3" THEN TL=1
964 IF ST$="6" THEN TL=3
970 PRINT:INPUT "NAME OF DATA FILE";F$
973 PCNT$=F$+".prn"
978 OPEN PCNT$ FOR OUTPUT AS #3
990 'PLOT AXES
995 SCREEN 2:CLS
1000 FOR Y=3 TO 162 STEP 3
1010    XC=80:W=2
1020    IF Y=30 OR Y=60 OR Y=90 OR Y=120 OR Y=150 THEN
XC=75:W=10
1025    LINE (XC,Y)-(XC+W,Y),1
1030 NEXT Y
1040 FOR X=80 TO 605 STEP 14
1050    YC=150:W=0
1060    IF X=150 OR X=220 OR X=290 OR X=360 OR X=430 OR
X=500 OR   X=570 THEN YC=151:W=4
1070    LINE (X,YC)-(X,YC-W),1
1080 NEXT X
1260  JJ$="BM+3,-7":KK$="BM+7,-7"
1270 IF TL=1 THEN 1472
1360  DRAW "BM284,155"
1410 DRAW N3$:DRAW JJ$:DRAW N0$
1460  DRAW "BM494,155"
1470 DRAW N6$:DRAW JJ$:DRAW N0$:GOTO 1500
1472  DRAW "BM284,155"
1474 DRAW N1$:DRAW JJ$:DRAW N0$
```

```
1476 DRAW "BM494,155"
1478 DRAW N2$:DRAW JJ$:DRAW N0$
1500 DRAW "S8;BM300,170"
1510 DRAW M$:DRAW JJ$:DRAW I$:DRAW JJ$:DRAW N$:DRAW
JJ$:DRAW U$:DRAW JJ$
1520 DRAW T$:DRAW JJ$:DRAW E$:DRAW JJ$:DRAW S$
1530 DRAW "A1;BM1,120":DRAW PER$:DRAW KK$:DRAW
     "BM1,105":DRAW O$:DRAW KK$:DRAW X$
1532 DRAW KK$:DRAW Y$:DRAW KK$:DRAW G$:DRAW KK$:DRAW
     E$:DRAW KK$:DRAW N$
1540 DRAW "S4;A0"
1541 DRAW "S4;BM49,27"
1542 DRAW N1$:DRAW JJ$:DRAW N0$:DRAW JJ$:DRAW N0$
1544 DRAW "BM51,57"
1546 DRAW N7$:DRAW JJ$:DRAW N5$
1548 DRAW "BM51,87"
1550 DRAW N5$:DRAW JJ$:DRAW N0$
1552 DRAW "BM51,117"
1554 DRAW N2$:DRAW JJ$:DRAW N5$
2000     '-----------------------------------------------
---------------     ----
2001 'COLLECT AND PLOT DATA
2002 KEY(3) ON
2010
MEASURE.VOLTS=138:SET.ERROR.CONTROL.WORD=75:GET.ERROR.CO
     DE=78
2015 DEF SEG=&H0:PCLSEG=PEEK(&H4FE)+256*PEEK(&H4FF):DEF
     SEG=PCLSEG
2040
TIME.INT=10:NUM.SEC=SAMP.TIM*60:ERROR.VALUE%=0:CH3%=3
2047 CALL SET.ERROR.CONTROL.WORD(ERROR.VALUE%)
2050 PRINT "HIT ANY KEY TO BEGIN":A$=INPUT$(1)
2055 PRINT "USE F3 TO STOP"
2060 TIME$="00:00:00"
2070 'TIMING LOOP
2080 S!=TIMER/TIME.INT
2090 Z!=S!-INT(S!):IF Z!<.0054 GOTO 2110  'Z!<.0015 FOR 30s
INTERVALS
2100 GOTO 2070
2110 CALL MEASURE.VOLTS(CH3%,VOLTS)
2120 CALL GET.ERROR.CODE(ERROR.VALUE%)
```

```
2130 IF ERROR.VALUE%<>0 THEN PRINT "** ACQUISITION ERROR
**":END
2135  X=INT(210/(TL*600)*TIMER+80):Y=INT(-12*VOLTS+150)
2137  PT=VOLTS*10
2140 WRITE #3,TIMER,PT
2150 PSET(X,Y)
2170 GOTO 2070
2180 END
5000      '-----------------------------------------------------
---------------      ----
5002 'Subroutine `LETTERS'
5004 A$="BR5BD3L5ND4U2E1R3F1D6"
5006 B$="ND3R4F1D1G1L4D4R4E1U2H1BD4BR1"
5008 C$="BR5BD1H1L3G1D5F1R3E1BD1"
5010 D$="ND7R4F1D5G1L4BR5"
5012 E$="NR5D3NR4D4R5"
5014 F$="NR5D3NR4D4BR5"
5016 G$="BR5L4G1D5F1R3E1U2L2BD3BR2"
5018 H$="D3ND4R5NU3D4"
5020 I$="R1NR1D7NL1R1"
5022 J$="BD5D1F1R3E1U6BD7"
5024 K$="D3NE3ND4F4"
5026 L$="D7R5"
5028 M$="ND7F3E3D7"
5030 N$="ND7F7NU7"
5032 O$="BD1D5F1R3E1U5H1L3G1BD6BR5"
5034 P$="ND3R4F1D1G1L4D4BR5"
5036 Q$="BD1D5F1R3E1U5H1L3G1BD4BR3F2"
5038 R$="ND7R4F1D1G1L4F4BR1"
5040 S$="BR5L4G1D1F1R3F1D2G1L4BR5"
5042 T$="R3NR3D7BR3"
5044 U$="D6F1R3E1U6BD7"
5046 V$="D4F3E3U4BD7"
5048 W$="D7E3F3U7BD7"
5050 X$="F3NE3D1NG3F3"
5052 Y$="F3NE3D4BR3"
5054 Z$="R7G7R7"
5056 N0$="BR4BD7E1U5H1L3G1D5F1R3BR1"
5058 N1$="BD1E1D7NL1R1"
5060 N2$="BD1E1R3F1D1G1L3G1D3R5"
5062 N3$="BD1E1R3F1D1G1NL3F1D2G1L3H1BR5BD1"
5064 N4$="BR5G5R5NR1NU5D2BR1"
```

```
5066 N5$="BR5L5D3R4F1D2G1L3H1BR5BD1"
5068 N6$="BR5BD1H1L3G1D5F1R3E1U2H1L3G1BD3BR5"
5070 N7$="BD1U1R5D7"
5072 N8$="BR1BD3H1U1E1R3F1D1G1L3G1D2F1R3E1U2H1BD4BR1"
5074 N9$="BR5BD3G1L3H1U2E1R3F1D5G1L3H1BR5BD1"
5075 PER$="D2R2U2L2BR7G7BR7U2L2D2R2"
5076      '-------------------------------------------------
---------------      ----
5080 RETURN
8000 SCREEN 0,0,0:END
8010 END
```

APPENDIX B: PROCESS DATA.

Table B-1. Biomass measurements for shake flask study run 1. Conditions: 27 °C, 125 mL flask, 150 rpm, 30 g/L of sucrose.

DAY	PCV (v/v) ±1 s	DRY CELL DENSITY (g/L) ±1 s	CONDUCTIVITY (mmhos) ±1 s
0	0.200 ± 0.017	2.1 ± 0.09	5350 ± 36.1
2	0.203 ± 0.022	3.0 ± 0.09	5448 ± 71.8
4	0.203 ± 0.017	3.5 ± 0.17	5425 ± 170.6
5	0.213 ± 0.032	3.9 ± 0.40	5230 ± 113.6
6	0.208 ± 0.047	4.3 ± 0.31	5020 ± 194.7
7	0.275 ± 0.019	6.0 ± 0.21	5010
8	0.300 ± 0.014	6.9 ± 0.26	4710 ± 14.1
9	0.350 ± 0.000	7.9 ± 0.11	4700 ± 28.3
10	0.505 ± 0.021	9.8 ± 0.15	
12	0.630 ± 0.037	11.0 ± 0.42	4230
13	0.655 ± 0.050	10.7 ± 0.17	4220
15	0.720 ± 0.028	10.2 ± 0.05	
17	0.760 ± 0.014	10.1 ± 0.06	
19	0.800 ± 0.001	10.7 ± 0.64	4180
20	0.910 ± 0.001	10.8 ± 0.28	4240

Table B-2. Biomass measurements for shake flask study run 2. Conditions: 27 °C, 125 mL flask, 150 rpm, 30 g/L of sucrose.

DAY	PCV (v/v) ± 1s	DRY CELL DENSITY (g/L) ± 1s	CONDUCTIVITY (mmhos) ± 1s
0	0.152 ± 0.030	2.3 ± 0.29	5240.0
1	0.180 ± 0.014	2.9 ± 0.10	5170.0 ± 141.4
2	0.180 ± 0.028	3.4 ± 0.08	4745.0 ± 388.9
3	0.185 ± 0.021	4.0 ± 0.24	4845.0 ± 374.8
5	0.253 ± 0.015	5.9 ± 0.14	4640.0 ± 297.0
7	0.323 ± 0.024	7.7 ± 0.62	4040.0
8	0.420 ± 0.034	9.4 ± 0.25	4175.0
9	0.470 ± 0.000	10.7 ± 0.08	4000.0 ± 84.9
11	0.705 ± 0.027	12.8 ± 0.69	
13	0.830 ± 0.039	11.3 ± 0.59	4350.0
14	0.815 ± 0.021	11.4 ± 0.10	
16	0.875 ± 0.037	12.2 ± 1.01	

Table B-3. Biomass measurements for shake flask study run 3. Conditions: 27 °C, 125 mL flask, 150 rpm, 30 g/L of sucrose.

DAY	PCV (v/v) ± 1s	DRY CELL DENSITY (g/L) ± 1s	CONDUCTIVITY (mmhos) ± 1s
1	0.22 ± 0.028	3.80 ± 0.196	5307 ± 882
2	0.17 ± 0.022	4.23 ± 0.331	5223 ± 754
3	0.24 ± 0.029	3.07 ± 0.422	5572 ± 450
4	0.24 ± 0.021	5.38 ± 0.598	4960 ± 250
5	0.28 ± 0.021	6.55 ± 0.318	4612 ± 262
6	0.37 ± 0.006	8.04 ± 0.071	4470 ± 110
7	0.44 ± 0.015	9.35 ± 0.338	4220 ± 271
8	0.50 ± 0.010	10.78 ± 0.244	3810 ± 104
9	0.66 ± 0.040	13.92 ± 1.154	3795 ± 262
10	0.82 ± 0.076	13.69 ± 0.794	3475 ± 160
11	0.81 ± 0.274	13.90	3640 ± 141
12	0.86 ± 0.025	12.89 ± 0.462	2858 ± 389
13	0.86 ± 0.012	13.30 ± 0.058	2765 ± 219
15	0.86	11.32 ± 0.820	2850 ± 56

Table B-4. Cell growth at different S_o values for shake flask study.
Conditions: 27 oC, 125 mL flask, 150 rpm

DAY	DRY CELL DENSITY (g/L) So = 20 g/L ± 1s	DRY CELL DENSITY (g/L) So = 30 g/L ± 1s	DRY CELL DENSITY (g/L) So = 40 g/L ± 1s
0	2.7 ± 0.12	2.1 ± 0.081	2.1 ± 0.08
1	2.8 ± 0.34		2.6 ± 0.06
2	3.8 ± 0.24	3.0 ± 0.09	3.6 ± 0.08
3	2.4 ± 0.04		
4	4.4 ± 0.30	3.5 ± 0.18	
5		3.9 ± 0.40	6.4 ± 0.22
6	5.7 ± 0.40	4.3 ± 0.31	7.0 ± 0.08
7	7.0 ± 0.25	6.0 ± 0.21	9.6 ± 0.81
8	7.9 ± 0.23	6.9 ± 0.26	10.5 ± 0.46
9	7.1 ± 0.07	7.9 ± 0.11	11.0 ± 0.37
10		9.8 ± 0.15	13.4 ± 1.32
12		11.5 ± 0.42	
13		10.7 ± 0.17	
14	7.9 ± 0.36		17.2 ± 0.71
15	7.7 ± 0.15	10.2 ± 0.05	16.1 ± 0.87

Table B-5. Biomass, % D.O., and pH data for bioreactor run 1.
Cultivation Conditions:
total liquid volume = 1600 mL 150 rpm
inoculum volume = 300 mL 27 °C
age of inoculum = 8 days paddle impeller
nitrogen flow = 1.14 L/min.
air flow = 0.7 L/min.

TIME (days)	DRY CELL DENSITY (g/L)	PCV (v/v)	CONDUCT. (mmhos)	%D.O.	pH
1	2.0	0.070	4.95	44.1	5.45
2	2.7	0.120	4.97	37.5	5.34
3	2.6	0.140	4.65	33.2	5.45
4	2.9	0.160		27.2	5.32
5	3.7	0.165	4.77	29.6	5.33
6	4.1	0.180	4.51	19.4	5.35
7	3.4	0.205	4.56	17.8	5.24
8	2.7	0.135	4.66	62.4	4.64
9	2.37	0.130	4.45	91.9	4.49
10	2.50	0.110	4.58	90.0	4.47

Table B-6. Growth rate data for bioreactor run 1.
Cultivation Conditions:
total liquid volume = 1600 mL 150 rpm
inoculum volume = 300 mL 27 °C
age of inoculum = 8 days paddle impeller
nitrogen flow = 1.14 L/min.
air flow = 0.7 L/min.

TIME (days)	Q_o No N_2 (mmole O_2/ hr)	k_La No N_2 (hr^{-1})	Q_o w/N_2 (mmole O_2/ hr)	k_La w/N_2 (hr^{-1})
1	0.863	5.821	0.831	5.606
2	0.841	5.840	0.883	5.943
3	0.922	4.140	1.084	5.860
4			0.386	2.000
5	0.649	3.695	0.732	3.376
6	0.665	3.273	0.338	1.714
7	0.582	3.181	0.451	2.049
8	0.241	3.420	0.691	6.416
9	0.005	2.294	0.345	9.495

Table B-7. Biomass, % D.O., and pH data for bioreactor run 2.
Cultivation Conditions:
total liquid volume = 1600 mL 150 rpm
inoculum volume = 300 mL 27 °C
age of inoculum = 8 days paddle impeller
nitrogen flow = 1.14 L/min.
air flow = 0.7 L/min.

TIME (days)	DRY CELL DENSITY (g/L)	PCV (v/v)	CONDUCT. (mmhos)	%D.O.	pH
1	1.84	0.110	5.36	54.8	5.75
2	1.84	0.080	5.40	65.6	5.51
3	1.83	0.110	5.49	60.5	5.44
4	1.67	0.078	5.40	70.6	5.48
5	1.61	0.086	5.40	86.2	4.83
6	1.50	0.066	5.36	85.3	4.57
7	1.50	0.065	4.82	78.4	4.49
8	1.60	0.048		86.6	4.43
9	1.58	0.060	4.73	87.4	4.37

Table B-8. Growth rate data for bioreactor run 2.
Cultivation Conditions:
total liquid volume = 1600 mL 150 rpm
inoculum volume = 300 mL 27 °C
age of inoculum = 8 days paddle impeller
nitrogen flow = 1.14 L/min.
air flow = 0.7 L/min.

TIME (days)	Q_o No N_2 (mmole O_2/ hr)	k_La No N_2 (hr^{-1})	Q_o w/N_2 (mmole O_2/ hr)	k_La w/N_2 (hr^{-1})
1	0.566	5.713	0.792	5.927
2	0.439	5.051	0.697	4.925
3	0.452	5.449	0.562	4.248
4	0.267	5.223	0.741	9.191
5	0.262	8.315	0.607	7.155
6	0.280	7.429	0.758	6.329
7	0.193	4.511	0.657	5.233
8	0.212	5.415	0.669	5.811
9	0.137	5.468	0.578	5.753

Table B-9. Biomass, % D.O., and pH data for bioreactor run 3.
Cultivation Conditions:
total liquid volume = 1800 mL 150 rpm
inoculum volume = 500 mL 27 °C
age of inoculum = 8 days paddle impeller
air flow = 0.8 L/min.

TIME (days)	DRY CELL DENSITY (g/L)	PCV (v/v)	%D.O.	pH
0.21	1.21	0.050	51.6	5.47
1.00		0.110	39.7	5.48
2.00	2.34	0.100	33.7	5.77
3.13	1.97	0.120	32.1	5.34
4.00	2.43	0.100	30.9	5.19
5.44	2.73	0.140	28.7	5.52
6.23	3.08	0.140	29.4	5.37
6.98	1.91	0.120	29.2	4.82
8.29	1.54	0.100	32.4	4.46
9.21	1.61	0.120	34.1	4.29

Table B-10. Growth rate data for bioreactor run 3.
Cultivation Conditions:
total liquid volume = 1800 mL 150 rpm
inoculum volume = 500 mL 27 °C
age of inoculum = 8 days paddle impeller
air flow = 0.8 L/min.

TIME (days)	Q_o NO N_2 (mmole O_2/ hr)	k_La No N_2 (hr^{-1})
0.21	0.053	0.513
1.00	0.015	1.146
2.00	0.182	1.500
3.13	0.178	1.496
4.00	0.177	1.518
5.44	0.223	1.710
6.23	0.176	1.341
6.98	0.074	1.079
8.29	0.023	0.167
9.21	0.007	0.045

Table B-11. Computer file name and process parameter summary for bioreactor run 1.

Program #	Trial #	File Name	Process Parameters
1	1A	BIO9-1A	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C
2	1B	BIO9-1B	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C
3	1C	BIO9-1C	N_2, paddle impeller, 0.7 L/min air flow, 27 °C
4	2A	BIO9-2A	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C
5	2B	BIO9-2B	N_2, paddle impeller, 0.7 L/min air flow, 27 °C
6	3A	BIO9-3A	N_2, paddle impeller, 0.7 L/min air flow, 27 °C
7	3B	BIO9-3B	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C
8	4A	BIO9-4A	N_2, paddle impeller, 0.7 L/min air flow, 27 °C
9	5A	BIO9-5A	N_2, paddle impeller, 0.7 L/min air flow, 27 °C
10	5B	BIO9-5B	N_2, paddle impeller, 0.7 L/min air flow, 27 °C
11	5C	BIO9-5C	N_2, paddle impeller, 0.7 L/min air flow, 27 °C

Table B-11 (Continued).

Program #	Trial #	File Name	Process Parameters
12	5D	BIO9-5D	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C
13	6A	BIO9-6A	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C
14	6B	BIO9-6B	N_2, paddle impeller, 0.7 L/min air flow, 27 °C
15	6C	BIO9-6C	N_2, paddle impeller, 0.7 L/min air flow, 27 °C
16	6D	BIO9-6D	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C
17	7A	BIO9-7A	N_2, paddle impeller, 0.7 L/min air flow, 27 °C
18	7B	BIO9-7B	N_2, paddle impeller, 0.7 L/min air flow, 27 °C
19	7C	BIO9-7C	N_2, paddle impeller, 0.7 L/min air flow, 27 °C
20	7D	BIO9-7D	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C
21	8A	BIO9-8A	N_2, paddle impeller, 0.7 L/min air flow, 27 °C
22	8B	BIO9-8B	N_2, paddle impeller, 0.7 L/min air flow, 27 °C

Table B-11 (Continued).

Program #	Trial #	File Name	Process Parameters
23	8D	BIO9-8D	NO N_2, paddle impeller, 0.7 L/min air flow, 27 °C
24	9A	BIO9-9A	N_2, paddle impeller, 0.7 L/min air flow, 27 °C
25	9B	BIO9-9B	N_2, paddle impeller, 0.7 L/min air flow, 27 °C
26	9D	BIO9-9D	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C
27	10A	BIO9-10A	N_2, paddle impeller, 0.7 L/min air flow, 27 °C
28	10D	BIO9-10D	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C

Table B-12. Computer file name and process parameter summary for bioreactor run 2.

Program #	Trial #	File Name	Process Parameters
1	1A	BIO10-1A	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
2	1B	BIO10-1B	N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
3	1C	BIO10-1C	N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
4	1D	BIO10-1D	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
5	2A	BIO10-2A	N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
6	2B	BIO10-2B	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
7	2C	BIO10-2C	N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
8	2D	BIO10-2D	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
9	3A	BIO10-3A	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
10	3B	BIO10-3B	N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
11	4A	BIO10-4A	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C.

Table B-12 (Continued).

Program #	Trial #	File Name	Process Parameters
12	4B	BIO10-4B	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
13	4C	BIO10-4C	N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
14	4D	BIO10-4D	N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
15	5A	BIO10-5A	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
16	5B	BIO10-5B	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
17	5C	BIO10-5C	N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
18	5D	BIO10-5D	N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
19	5E	BIO10-5E	N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
20	6A	BIO10-6A	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
21	6B	BIO10-6B	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
22	6C	BIO10-6C	N_2, paddle impeller, 0.7 L/min air flow, 27 °C.

Table B-12 (Continued).

Run #	Trial #	File Name	Process Parameters
23	6D	BIO10-6D	N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
24	7A	BIO10-7A	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
25	7B	BIO10-7B	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
26	7C	BIO10-7C	N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
27	7D	BIO10-7D	N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
28	8A	BIO10-8A	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
29	8B	BIO10-8B	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
30	8C	BIO10-8C	N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
31	8D	BIO10-8D	N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
32	9A	BIO10-9A	No N_2, paddle impeller, 0.7 L/min air flow, 27 °C.
33	9B	BIO10-9B	N_2, paddle impeller, 0.7 L/min air flow, 27 °C.

Table B-13. Computer file name and process parameter summary for bioreactor run 3.

Program #	Trial #	File Name	Process Parameters
1	1A	BIO13-1A	No N_2, paddle impeller, 0.8 L/min air flow, 27 °C.
2	1B	BIO13-1B	No N_2, paddle impeller, 0.8 L/min air flow, 27 °C.
3	1C	BIO13-1C	No N_2, paddle impeller, 0.8 L/min air flow, 27 °C.
4	2A	BIO13-2A	No N_2, paddle impeller, 0.8 L/min air flow, 27 °C.
5	2B	BIO13-2B	No N_2, paddle impeller, 0.8 L/min air flow, 27 °C.
6	3B	BIO13-3B	No N_2, paddle impeller, 0.8 L/min air flow, 27 °C.
7	3C	BIO13-3C	No N_2, paddle impeller, 0.8 L/min air flow, 27 °C.
8	4A	BIO13-4A	No N_2, paddle impeller, 0.8 L/min air flow, 27 °C.

Table B-13 (Continued).

Program #	Trial #	File Name	Process Parameters
9	4B	BIO13-4B	No N_2, paddle impeller, 0.8 L/min air flow, 27 °C.
10	5A	BIO13-5A	No N_2, paddle impeller, 0.8 L/min air flow, 27 °C.
11	6A	BIO13-6A	No N_2, paddle impeller, 0.8 L/min air flow, 27 °C.
12	6B	BIO13-6B	No N_2, paddle impeller, 0.8 L/min air flow, 27 °C.
13	7A	BIO13-7A	No N_2, paddle impeller, 0.8 L/min air flow, 27 °C.
14	7B	BIO13-7B	No N_2, paddle impeller, 0.8 L/min air flow, 27 °C.
15	8A	BIO13-8A	No N_2, paddle impeller, 0.8 L/min air flow, 27 °C.
16	9A	BIO13-9A	No N_2, paddle impeller, 0.8 L/min air flow, 27 °C.

Table B-13 (Continued).

Program #	Trial #	File Name	Process Parameters
17	10A	BI13-10A	No N_2, paddle impeller, 0.8 L/min air flow, 27 °C.

APPENDIX C: PROTOCOLS.

Protocol for callus culture maintenance.

1. Prepare about 1 L medium with composition shown below:
   - MS nutrient base Sigma M5519, 4.4 g/L
   - 5.7 µM IAA (Indole-3-Acetic Acid, MW+ 175.2) 1.0 mg/L
   - 8.o g/L agar
   - 30 g/L sucrose
   - adjust pH to 5.8 - 6.0
2. Autoclave medium at 15 psig, 250 °F for 15 minutes to dissolve agar.
3. Pour 30 mL of medium into 125 mL flasks.
4. Seal flask by placing cotton plugs, covered in cheese cloth, on top. Cover plugs with foil for autoclaving.
5. Autoclave at 15 psig, 250 °F for 30 minutes.
6. Let cool until agar solidifies.

Perform steps 7 through 11 in laminar flow hood.

7. Using flame sterilized forceps, remove callus from flask and set in sterile petri dish.
8. Using flame sterilized knife, slice off three loose, small white (friable) callus portions.
9. Transfer friable callus onto fresh medium. Evenly distribute the three pieces in the 125 mL flask.
10. Flame the top of the flask, seal with cotton plug, and place in a dark cabinet at 23 °C for three weeks. After three weeks, repeat steps 1 through 10.

Protocol for initiation of suspension cultures from callus cultures.

1. Prepare suspension culture medium as indicated in Suspension Culture Maintenance protocol.

2. Pour 30 mL medium into 125 mL flasks.

3. Seal flask with cotton plugs, autoclave at 15 psig, 250 °F for 30 min, and cool to room temperature.

Perform steps 4 through 7 in laminar flow hood.

4. Transfer callus cultures into petri dish as dictated in Callus Culture Maintenance protocol.

5. Cut about 2 grams of friable callus pieces off three callus cultures and transfer with knife into liquid medium.

6. Flame flask and seal with plug.

7. Place flask on shake at 150 rpm and 27 °C.

Protocol for suspension culture maintenance.

Subculturing of *Nicotiana tabacum*:

1. Prepare about 1 L medium with composition shown below:
   - MS nutrient base Sigma M5519, 4.4 g/L
   - 2.3 μM 2,4-D (2,4,-Dichlorophenoxyacetic Acid, MW= 221 g/mole) 0.5 mg/L
   - 0.14 μM Kinetin (6-Furfurylaminopurine, MW= 215.2 g/mole) 30 g/L
   - 30 g/L sucrose
   - adjust pH to 5.8-6.0

2. Pour medium into Erlenmeyer Flask

Flask size	Amount of Medium
125 mL	30 mL
250 mL	50 mL
500 mL	100 mL

3. Seal flask by placing cotton plugs, covered in cheese cloth, on top. Cover plugs with foil for autoclaving.

4. Autoclave at 250 oF, 15 psig for 30 minutes.

5. Let medium cool to room temperature

Perform steps 6 and 7 in a laminar flow hood.

6. Using a Kimax 10 mL pipet (tip diameter = 1/8") transfer cell culture from old flask to sterilized medium.

Flask Size	Amount of cells transfered
125 mL	10 mls
250 mL	20 mls
500 mL	40 mls

* When transfering cultures from 125 mL flask to 500 mL flask, cultures were poured directly from the 125 mL flask into the 500 mL flask after both tips were flame. The 500 mL flask was then flamed again after transfer and sealed.

7. Flame the flask opening and re-seal with cotton plug.

Protocol for suspension culture maintenance (continued).

8. Remove foil from plugs and place flask on rotor shaker

Flask Size	Rotary Shaker Speed
125 mL	150 rpm
250 mL	130 rpm
500 mL	115 rpm

Protocol for cell density measurements.

Packed-Cell Volume (PCV):

1. Remove flask from shaker.

2. Swirl flask and pour 10.0 mL of cell & slurry into 15 mL centrifuge tube (conical, plastic). Cover tube with parafilm.

3. Insert centrifuge tube into rubber stopper insert; fit snugly into centrifuge tube shield, and load into centrifuge tube shield, and load into centrifuge rotor. Balance rotor with two samples. Centrifuge at speed setting 50 (2000 rpm, 523g) for 15 min. Centrifuge: IEC Centra 4B; Rotor: IEC 320

4. Pastuer pipet supernatant from centrifuge tube into 10 mL graduated cylinder; measure volume of culture liquid (mL). To determine PCV%:

   PCV% = (10 mL - mL culture medium)/10 mL slurry *100

5. Transfer liquid to glass vial and store frozen.

Dry Cell Weight Measurement:

6. Weigh a pre-dried disk (55 mm) of Whatman filter paper #1 and place on Buchner funnel. Decant packed cell mass from centrifuge tube by squirting pellet with distilled water; pour slurry onto Buchner funnel and filter under vacuum for 10 min.

7. Place filter paper & wet cell cake in oven. Dry at 60 °C (setting = 1.8 on VWR 1305U oven) for 48 hr.

8. Weigh dried cell mass to precision of +/-0.0001g. To determine cell density:

   cell density = (g dry cell + F.P. - g dry F.P.)/10 mL * 1000 mL/L

Protocol for conductivity measurements.

Calibration of Meter:

1. Warm up the Markso ElectroMark Conductivity meter for approximately 1.5 hours.

2. Prepare various molar solutions of potassium nitrate (MW=101.1) at 21 °C. Molarities corresponding to corresponding values in a CRC are recommended: 0.94M, 1.38M, and 1.747M.

3. Let the potassium nitrate solution come to room temperature, or the temperature at which the samples ate to be measured at.

4. Place the solutions into Fisher brand glass vials, size 2DR, 7.4 mls.

5. Place the conductivity probe into the vial at a submerged length of 2 cm.

6. Allow approximately two minutes for the probe to equilabrate, then record measurement.

7. Repeat the run for each potassium nitrate solution.

8. Average the values for each run and plot a curve of molarity versus conductivity. A linear equation should result.

Sampling:

1. Use the supernate obtained after centrifuging a culture sample. The details for obtaining samples can be found in the protocol for cell density measurements.

2. All samples are brought to the temperature corresponding to the previous made calibration curve (21 °C).

Protocol for conductivity measurements (continued)

3. The probe is then submerged 2 cm. into the sample , which should be in a Fisher brand glass vial size 2DR, 7.4 mL.

4. Allow two minutes for the sample to equilabrate and record conductivity values. Also be sure to record the ambient temperature.

Protocol for plant cell culture inoculation of the VirTis 2 L stirred-tank bioreactor.

Sterilizing Reactor:

1. Wash reactor and parts with soap and water

2. Soak reactor and parts with 20% Clorox for 10 hours.

3. Rinse reactor and parts thoroughly with distilled water three times.

4. Fully assemble reactor and autoclave (215 °F, 15 psig), on slow exhaust, for one hour.

5. Pour in 1.2 L medium (formula found in Table 2) into reactor.

6. Re-seal reactor and place in autoclavable pan. Enclose the entire apparatus within a brown paper bag.

7. Transfer sealed pan to autoclave, and autoclave the apparatus for fifty minutes . Make sure exhaust is set to slow.

8. Place the entire autoclaved apparatus in a laminar flow hood.

9. Remove the paper bag from the pan and inspect reactor for any leaks. Leaks may occur because of: loose tubing, punctures in the tubing, loose fittings, or a filter came off during autoclaving. If leaks are present, repeat steps 3 through 8.

Inoculation:

10. After the reactor has cooled to touch, remove reactor from the pan. Replace the electrolyte in the DO probe (about 5 mL of the Galvanic O_2 filling solution.)

    Galvanic O2 filling solution (by volume):

    60% Ethylene Glycol

    40% 0.5 M NaOH

Protocol for plant cell culture inoculation of the VirTis 2 L stirred-tank bioreactor (continued).

11. Set the temperature of the medium to 27.0 °C, the rpm's to 150, and the air flow rate to 0.7 LPM.

12. Saturate the medium and bring the temperature to 27 °C by sparging in prehumidified air.

13. Electronically zero the probe, and then set 100% to the air-saturated medium.

14. Wash all exposed parts surrounding the probe on the reactor with a 95% ethanol solution.

15. Remove the probe being careful not to touch any surfaces. Hold probe with one hand, and with the other insert an autoclaved-flame sterilized funnel, using pre-flamed forceps, into the D.O. probe port.

16. Pour the inoculum from three 500 mL flasks containing 140 mL of 8 day old tobacco cultures, into the reactor through the funnel. Prior to pouring out the contents in the 500 mL flask, flame the lip of the flask.

17. Remove the funnel and screw in the D.O. probe until completely sealed.

Protocol for plant culture oxygen mass transfer measurements in the VirTis 2 L stirred-tank bioreactor.

Set up Computer for Data Acquisition:

1. Turn on PC. Type
   C:\>bio
   to load up BASIC program for data acquisition.

2. Once program is loaded, select 60 minutes acquisition time.

3. Type in data file name (no .extension). A ".prn" extension is added to filename; file is stored in C:\O2DAT directory.

4. When ready to start, hit space bar once to start data acquisition. Let program run for 3 minutes to establish the baseline.

Oxygen Transfer Measurement:

5. Turn nitrogen on and let it flow over reactor medium at a rate of 0.7 L/min until equilibrium is reached.

6. Turn air pump switch to OFF. Watch dissolved oxygen concentration (expressed as % of saturation, 0-100) decrease linearly with time.

7. When dissolved oxygen concentration level hits 30.0 (usually within 30 minutes), turn air pump switch to ON (red light) and adjust air flow to desired level (typically o.7 L/min). Watch dissolved oxygen concentration increase with time, steadily approaching the baseline level. At 60 minutes, the program will end acquisition.

8. Perform steps 6 and 7 three times.

Protocol for plant culture oxygen mass transfer measurements in the VirTis 2 L stirred-tank bioreactor (continued).

Sampling and Program Shut-Down:

9. Clear sample line:
    a. Make sure flexible hose on sampling line is clamped.
    b. Screw 10 mL glass vial onto sampling port tightly, then unscrew one-half turn.
    c. Press in bulb fully, then screw vial on tightly.
    d. Unclamp flexible hose; 10 mL of bioreactor contents will empty into vial within one second.
    e. Repeat steps 6c to get 20 mL total sample.
    f. Clamp flexible hose immediately.
    g. Cap vial and store in freezer.

10. Shut data acquisition program. Hit F3 to end program.

11. When steps 1 through 10 have been completed, turn off nitrogen.

APPENDIX D: CALIBRATION DATA.

Table D-1. Calibration data for Markso ElectoMark conductivity meter. Conditions: Potassium nitrate used as standard, reference values reported at 20 °C, standard at 21.5 °C.

KNO_3 (moles/L)	REFERENCE 20 °C (mmhos)	STANDARD 21.5 °C (mmhos)
0.20	20.1	25.6 ± 0.5
0.94	79.5	82.2 ± 2.2
1.38	123.4	107.5 ± 5.8
1.75	131.0	116.5 ± 3.0

Table D-2. Calibration data for IEC Centa-4B Centrifuge, Rotor IEC 320.

RPM	SPEED SETTING
1130	40
1520	45
1920	50
2330	55
2740	60
3420	62.5
3960	65
3980	70
4460	75
5010	80

Table D-3. Calibration data of sugar concentrations obtained from the HPLC.

COMPONENT	RETENTION TIME, min (± 1s)	RESPONSE FACTOR μV-sec/ μg (± 1s)
Sucrose	10.40 ± 0.07	2.67 E 05 ± 1.6 E 03
Glucose	12.53 ± 0.07	2.71 E 05 ± 1.6 E 03
Fructose	16.72 ± 0.18	2.43 E 05 ± 1.5 E 03
Sorbitol	35.67 ± 0.24	2.71 E 05 ± 1.6 E 03

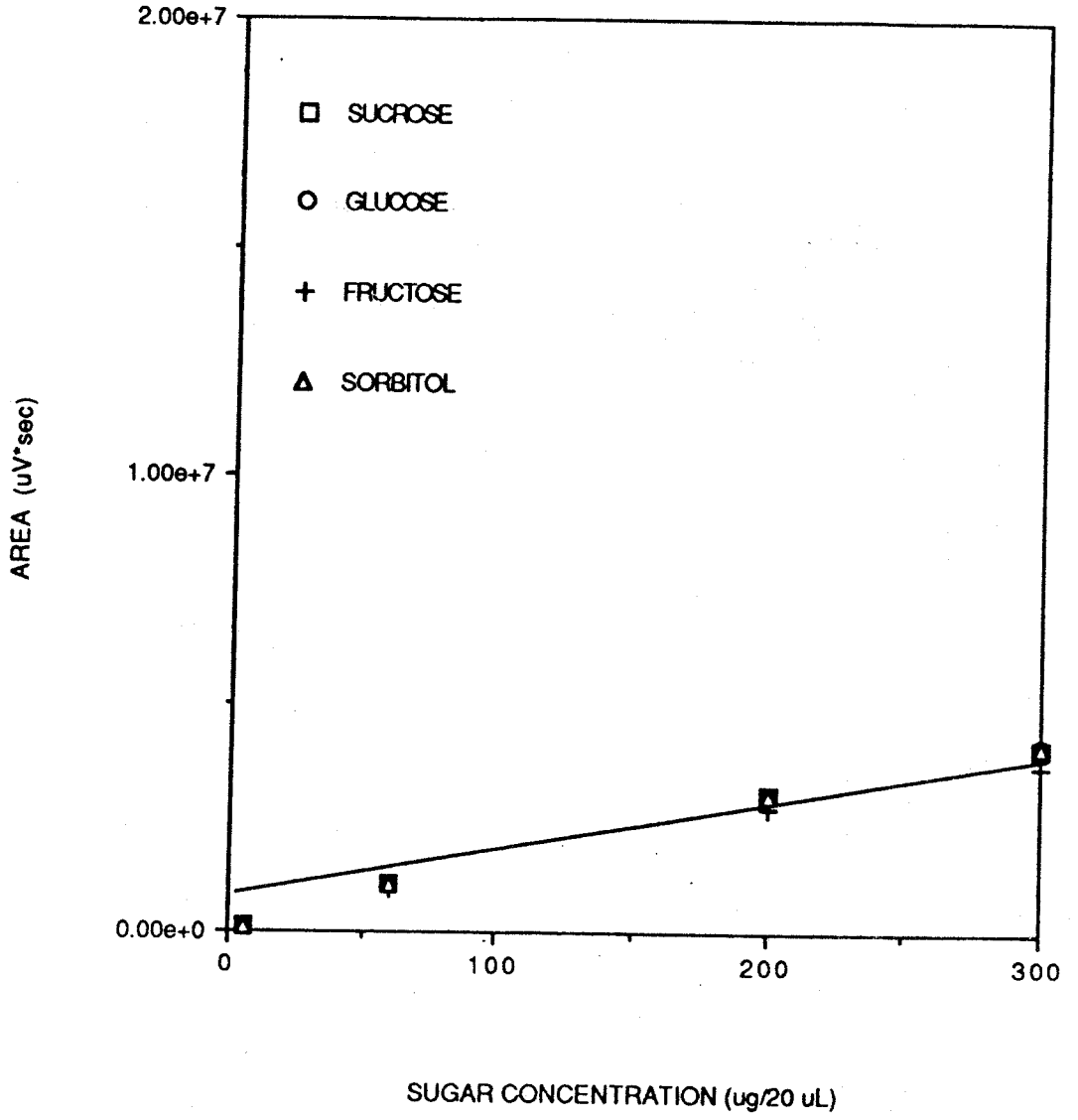

Figure D-1. Calibration plot for sugar concentrations obtained from the HPLC.